PATENT
Docket No. 559662000101

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Marian Christopher

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re Patent Application of:

Bruce J. ROSER

Serial No.: 09/888,734

Filing Date: June 25, 2001

For: DRIED BLOOD FACTOR COMPOSITION COMPRISING TREHALOSE

Examiner: Francisco Chandler Prats

Group Art Unit: 1651

BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant hereby appeals from the final rejection of claims 14-16 and 20-22, all claims pending in the above-referenced application. In accordance with 37 C.F.R. § 1.192, this Brief, along with the Appendixes, is accompanied by the required fee. A Notice of Appeal was filed action on 28 February 2005, thus setting a date for filing of the Brief of 28 April 2005. A petition for an extension of time of three (3) months until 28 July 2005 is enclosed, along with the required fee. Appellant respectfully requests reversal of the Examiner's decision to reject claims 14-16 and 20-22.

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1. Real Party in Interest

The parties having an interest in the present invention are the assignee, Quadrant Holdings Cambridge Limited and its exclusive licensee, Baxter Healthcare Corporation.

2. Related Appeals and Interferences

To appellant's knowledge, there are no other appeals or any interferences known to him or his legal representative or assignee that will directly affect or be directly affected by or have a bearing on the Board's decision in the present Appeal.

3. Status of Claims

Claims 14-16 and 20-22 are currently pending and on appeal. Claims 1-13, originally filed with the application, have been canceled and claims 17-19, which were added in a Response mailed 25 September 2002 to an Office action mailed 25 March 2002, have also been canceled.

Thus, claims 1-13 and 17-19 have been canceled and claims 14-16 and 20-22 are pending and appealed.

4. Status of Amendments

No amendments to the claims were proposed in response to final rejection.

5. Summary of Claimed Subject Matter

Claim 14 is the only independent claim. It is directed to a method for preparing a stable dried composition of "native" Factor VIII. The composition contains a stabilizing amount of trehalose but does not contain a stabilizing amount of albumin. The method comprises freeze-drying an aliquot of an aqueous solution of Factor VIII containing trehalose and free of albumin.

The specification as filed does not contain page or line numbers; accordingly, attached to this Brief as an Exhibit is a copy of the specification with the appropriate numbers inserted so as to facilitate reference to the specification.

The concept that the Factor VIII composition is prepared by freeze-drying in the presence of trehalose and in the absence of a stabilizing amount of albumin is set forth on page 1 of the specification at lines 11-15 which state that it is customary to stabilize freeze-dried Factor VIII by the addition of human serum albumin and lines 20-23 state that it has been found that if trehalose is used to stabilize a blood factor product (such as Factor VIII) the product can be dried, with or without freezing, in the absence of human serum albumin (HSA). Avoiding the presence of albumin has the advantage of avoiding any possible risk of contamination with viruses or prions. Lines 23-24 conclude that according to the invention there is provided a stable, dried blood factor composition containing a stabilizing amount of trehalose in the absence of albumin. Page 2 of the specification, at lines 13-15, states that the dried composition may be obtained by drying an appropriate *solution* of the blood factor containing the trehalose and other desired components. Line 18 indicates that the methods of drying include freeze-drying. It is believed that the word "aliquot" is inherent in the description of such a procedure. Nevertheless, this word is used in connection with one drying method at least on page 1 at line 29. Example 3, which describes freeze-drying, indicates that "samples" are separately frozen; essentially synonymous with aliquots.

The word "native" Factor VIII is not defined in the specification, but this designation was suggested during prosecution of the parent application for the purposes of distinguishing Factor VIII as it would be prepared recombinantly or isolated from natural sources from "activated" Factor VIII which is described in one of the documents made the basis for rejection –

U.S. patent 5,824,780. "Activated" Factor VIII is a different protein, which is made by treating native Factor VIII with certain proteolytic agents, such as thrombin. It is clear from the specification that no such treatment is employed in the Factor VIII either used in the examples or referred to in the specification. Thus, the designation of the "native" Factor VIII simply makes explicit what is clearly described in the specification.

Claims 15 and 16, dependent on claim 14, provide that the Factor VIII may be either plasma-derived or recombinant. This is supported in the specification on page 1, lines 4-7.

Claims 20-22 are dependent on claims 14-16, respectively, and simply require that the aqueous solution that is freeze-drying further contains histidine. This is supported in the specification on page 1, at line 33. Histidine is also included in the formulations that are dried as described in Examples 1-3.

Thus, as set forth in the specification, the invention lies in the ability to freeze-dry aqueous solutions of Factor VIII in the presence of trehalose as a stabilizing agent, thus eliminating the requirement to employ albumin for this purpose.

The dependent claims will not be argued separately.

6. Grounds of Rejection to be Reviewed on Appeal

Claims 14-16 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Curtis, *et al.* (U.S. patent 5,824,780) in view of Livesey, *et al.* (U.S. patent 5,364,756).

Claims 14-16 and 20-22 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Curtis, *et al.* (U.S. patent 5,824,780) in view of Livesey, *et al.* (U.S. patent 5,364,756) as applied to claims 14-16 and further in view of Bhattacharva, *et al.* (U.S. patent 5,288,853). This rejection will not be separately argued, and claims 20-22 stand or fall with the decision on claims 14-16.

7. **Argument**

A. The rejection of claims 14-16 over Curtis in view of Livesey.

i) The teachings of Curtis combined with Livesey do not suggest the invention taken as a whole

The combination of Curtis with Livesey, even assuming motivation to do so, neither results in, nor suggests, the invention as claimed. Independent claim 14 contains a multiplicity of elements/limitations. It is directed to a method for *freeze-drying* an aliquot of a *solution* containing *native Factor VIII* in the presence *of a stabilizing amount of trehalose and in the absence of a stabilizing amount of albumin.* As will be discussed further below, the method solves a long-standing problem – to avoid the necessity of including albumin in Factor VIII compositions, as previously found necessary to stabilize them.

In order for the combination of documents cited to render the claimed subject matter obvious, the combination must not only suggest each and every claim limitation, but must also suggest the combination of all elements. As will be shown in greater detail below, the focus of the current rejection is on a single element of the claim – the use of trehalose as a cryoprotectant (whereas this is not what the claim is directed to), not the combination of elements that constitutes the invention as claimed. Neither of the cited documents even discloses freeze-drying a *solution* of *native Factor VIII.* The only document (Livesey) that describes a preservation process using trehalose in the absence of albumin does so in the context of a *suspension* of particulate biological material (polio virus) and even then fails to show efficacy of trehalose as a stabilizer. Moreover, as is explained in more detail below, the process in the relevant part of Livesey (namely, Example 5) involves a particular combination of (i) nebulizing a bulk suspension of the material (instead of an aliquot of a solution) and (ii) molecular distillation.

In the paragraphs below, each document is discussed in detail prior to summarizing applicant's position that the combination of the teachings of each document fails to disclose the invention taken as a whole.

The primary document, Curtis, is concerned with a method for preparing a purified and stable *activated* Factor VIII. For the reasons set forth below, any teaching regarding how activated Factor VIII might be stabilized when freeze-dried is irrelevant to the behavior of native Factor VIII. Curtis contains *no* teachings regarding preparing a freeze-dried form of native Factor VIII.

In column 5, lines 40-43 and column 6 lines 33-36 (which contain the only mentions of freeze-drying (lyophilization) in Curtis), it is stated, respectively, that "following the preparation and stabilization of the activated Factor VIII the protein can be lyophilized and stored at reduced temperatures..." and that "Lyophilization following activation, optionally followed by storage at a reduced temperature, such as –80°C, is also effective in producing a stable activated Factor VIII preparation". Thus, even as to activated Factor VIII, these passages teach away from the concept of the ability to prepare a composition that is stable without the need for refrigeration. The composition in accordance with the claimed method is stable at room temperature in the absence of albumin. (See the instant specification p. 1, 4th paragraph.)

In Curtis' Example 1, which is the only example that employs *native* Factor VIII, it is simply stated that Factor VIII is dialyzed and then treated with thrombin to form activated Factor VIII. The remaining examples deal entirely with activated Factor VIII.

It should be apparent simply from the fact that native Factor VIII must be treated with thrombin to obtain activated Factor VIII that the result would be a different composition. In addition, the considerable differences in characteristics and behavior between native and

activated Factor VIII have been explained in detail in the record and declarations from experts in the field have attested to this (see Evidence Appendix). Appellant appreciates that these declarations have been considered by the Examiner.

The Examiner's response to these declarations in an Advisory Action mailed 17 December 2004 is that rather than these differences teaching away from the claimed invention:

> The exact opposite is true. The fact that trehalose can be used to preserve both native and activated Factor VIII demonstrates that trehalose is recognized by the art as being a cryoprotectant suitable in a number of varied applications. The cited prior art clearly recognizes that the claimed agent, trehalose, is an established cryoprotection agent, which can be used to preserve proteins such as Factor VIII, as well as intact cells and other biological materials when these materials are saved by freeze-drying for future use. See the entire disclosure of Livesey. The art-recognized broad applicability of trehalose in cryopreservation methods bolsters rather than undermines the holding of *prima facie* obviousness.

These statements underline the error inherent in the rejection. Claim 14 is not directed merely to the use of trehalose as a cryoprotectant. The Examiner appears to have ignored in this context the requirement of 35 U.S.C. § 103* that a patent should be denied only if "the differences between the subject matter sought to be patented and the prior art are such that the subject matter *as a whole* would have been obvious at the time the invention was made." Clearly the invention has not been considered as a whole. The claim requires not only that trehalose be used, but that it be used in the absence of albumin in the freeze-drying process to stabilize native Factor VIII specifically and that this freeze-drying be conducted on an aliquot of a solution of the

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* The Examiner does quote the section of the statute in the Advisory Action to dispute applicant's alleged "urging that a specific format is required in obviousness determination." Applicant merely urges that, in addition to showing that a combination of documents suggests the invention, there must also be a rationale for a motivation to combine them.

native Factor VIII, instead of a frozen, nebulized powder, as in Livesey. (Livesey is further described below.) The recognition that trehalose is, among many others, a recognized cryoprotectant does not defeat the patentability of this combination of elements.

Even the sub-combination of Factor VIII freeze-dried in the presence of trehalose and in the absence of albumin (independent of whether a solution aliquot is used and whether activated or native Factor VIII is referred to) is nowhere taught or suggested in the documents cited by the Examiner. Livesey is discussed below. Curtis fails to teach anything at all about the freeze-drying of native Factor VIII. Even in suggesting that *activated* Factor VIII can be lyophilized, there is no suggestion in Curtis that trehalose be used in the absence of albumin. The lines immediately preceding the only mention of lyophilization in Curtis, at col. 5, lines 34-35, simply state "Examples of stabilizers include albumin, sucrose, maltose, glycine and trehalose." Thus, not only does Curtis fail to suggest freeze-drying of native Factor VIII at all, Curtis suggests lyophilization of activated Factor VIII without indicating that it is done in the presence of trehalose and the absence of albumin. Even if Curtis were combined with a document that teaches freeze-drying of native Factor VIII, Curtis cannot be used to suggest this combination of factors required by the claim.

Nevertheless, the Examiner takes the position that these deficiencies are overcome by Livesey which, as stated by its abstract, relates to "methods, apparatus and solutions for cryopreserving microscopic biological materials for biologically extended periods of time." In support of the position that trehalose can be used as a cryoprotectant in the absence of albumin, the Examiner points to Example 5. But Example 5 has nothing to do with Factor VIII. It is directed to the preservation and storage of oral polio vaccine which contains viral particles; such viral particles do not need the presence of albumin in order to be stable.

In addition, it is necessary to understand the specialized nature of the process that is disclosed in Livesey.

Livesey describes a two-step process that involves supercooling of a nebulized suspension followed by either freeze-drying or molecular distillation. This two-step process is contrasted by Livesey with conventional freeze-drying. In the conventional freeze-drying process, the solution of the material is frozen, generally to no lower than -70°C, the ice is sublimed and then any remaining water is desorbed. The ice that is formed in the freezing process has hexagonal crystals. (Col. 4, lines 34-37.)

In the nebulizing, supercooling and subsequent drying process that was invented by Livesey, a bulk suspension (including emulsions) of the material is nebulized and very rapidly cooled to a very low temperature, *i.e.*, less than -160°C (see Col 4 lines 21-22). This is much lower than the -70°C temperatures typically used for freeze-drying, and can be achieved only by nebulizing the material into very fine droplets by a special process set out at Col. 4 lines 17-22 and in more detail at Col. 5 line 48 to Col. 7 line 31, from which it is clear that only bulk liquids, not individual aliquots, can be used. The very rapid cooling to the extremely low temperature results in formation of amorphous phase water, cubic ice crystals and hexagonal crystals (Col. 14, lines 7-21). The frozen powdered sample is then transferred to another container and dried either by freeze-drying or molecular distillation. (Col. 4, lines 22-50.).

Livesey also distinguishes between freeze-drying and molecular distillation (Col. 3, lines 17-18), but in any case, the freeze-drying and molecular distillation occur only after supercooling of the nebulized microdroplets.

Thus, the Livesey process does not employ freeze-drying of any kind of aliquot or freeze-drying of any sample starting from room temperature. Freeze-drying and molecular distillation in Livesey occur only after nebulization and supercooling.

One can see that this specialized two-step process of Livesey is reflected in Example 5 thereof (the only example involving trehalose): at Col. 23, lines 45-47, the bulk suspension of viral particles is "rapidly frozen using the nebulizing device previously described", and then, at Col. 43, lines 52-55, the nebulized samples are "dried by molecular distillation". Hence, there is no disclosure of freeze-drying an *aliquot* of a *solution* of the material.

Furthermore, the process was performed not on a solution, as required by the claims, but on a suspension of the viral particles. And finally, the example fails even to show that trehalose is an effective stabilizer in the exemplified process:

According to Example 5, the viral samples were resuspended in three different solutions "until fully *suspended*" to form a "cryosolution." These three solutions in which the viral samples were suspended were: 1) 1 M MES buffer; 2) 1 M Tris buffer; and 3) 250 mM trehalose and 1 M Tris buffer. It should be clear, both from this wording and from the nature of the viral particles themselves, that the particles are *suspended*, not dissolved, in the cryosolution.[†]

Thus, Example 5 relates to a bulk suspension of viral particles (clearly very different from an aliquotted solution of native Factor VIII) and tests the ability of these viral particles (not

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[†] The Examiner has contested this point, arguing that suspensions and solutions are indistinguishable as concepts. Applicant has supplied a multiplicity of definitions establishing facts to the contrary. As noted in the defined terms, solutions are homogeneous mixtures; suspensions are not. Reference in the Stedman "solution" definition to "dispersions" is not probative of any overlap, but rather indicates reference to a comparison composition to be distinguished. (See Evidence Appendix).

Factor VIII) to retain activity after molecular distillation. The results shown in this example are reproduced below.

Solution in which the viral particles are suspended	Serotype I	Serotype II	Serotype III
MES buffer alone	4.45	4.45	4.45
Tris buffer alone	5.41	4.94	6.08
250 mM Trehalose in Tris buffer	4.87	4.60	4.59
Control	7.02	6.18	7.13

These results are reported as TCD_{50}'s (*i.e.*, the therapeutic dose concentrations that are 50% effective) although actual units are not given. It is nonetheless clear that a lower number represents higher activity. As shown by these results, trehalose is no more effective than MES buffer alone as a cryoprotectant/stabilizer and not much better than Tris buffer alone. Neither buffer is considered a cryoprotectant or stabilizer. Thus, these results do not establish that trehalose alone is a suitable cryoprotectant/stabilizer – it appears to be no more effective than buffer.

It should also be noted that, in the Office action mailed September 1, 2004, in which the Examiner set forth his reasoning in more detail than in the recent Advisory Action, he stated (on page 4) that Livesey taught that

> "trehalose is one of a number of agents particularly suited for protein protection in freeze-drying procedures, and albumin is not".

But there is no disclosure in Livesey that albumin is not suitable for protecting proteins during freeze-drying. Indeed, albumin is specifically mentioned as being a suitable cryoprotectant (along with either trehalose or sorbitol) at column 9, lines 33 to 39.

All of the foregoing having been said, the real problem with Example 5 is that it is irrelevant to Factor VIII.

The Livesey process may well be suitable for preparing storable preparations of mammalian cells (Examples 1 to 3), erythrocytes (Example 4) and an oral polio vaccine (Example 5). None of these compositions needs to have the same degree of sterility as is required for an intravenously injectable product such as Factor VIII. The Livesey process would not be suitable for preparing storable preparations of an intravenously injectable protein such as Factor VIII, because it would be impractical to preserve sterility and to ensure a consistent dose in the final container in the context of a process that involves nebulizing a bulk suspension to form a frozen powder and then transferring the powder to the final container. The person skilled in the art of preparing intravenously injectable preparations would be aware of this and would view the disclosure of Factor VIII in amongst a list of cells, viruses and vaccines (Col. 4, lines 58-62) as simply inappropriate.

Moreover, the record in this prosecution is replete with evidence that it was believed necessary in the case of native Factor VIII specifically to include albumin as a stabilizing agent. (See Evidence Appendix.) This is not the case for oral polio vaccine. The requirement for the inclusion of serum albumin as a stabilizer as a problem to be solved is common to blood factors, such as native Factor VIII. Therefore, an example relating to freeze-drying of viral particles which never were believed to require albumin for stabilization in the first place is irrelevant to the invention as claimed.

The application itself is clear that this is the problem to be solved,

> Blood factor products such as Factor VIII are highly delicate, unstable proteins. They are usually supplied in the form of frozen solutions in an appropriate buffer or more generally as freeze-dried powders. Even the freeze-dried powders must be kept cold during storage. In order to stabilize the freeze-dried material, commercial products contain a stabilizing protein in particular human serum albumin (HSA).

This statement outlines the problem to be solved; this problem does not exist with respect to viral vaccine compositions. Therefore, an example showing freeze-drying of a vaccine composition in the presence of trehalose and in the absence of albumin (where trehalose is not effective, in any event, as a cryoprotectant/stabilizer) fails to suggest the invention as claimed. And, the product had to be "kept cold during storage", whereas the product of the present invention is stable without refrigeration.

Independent of this example, which is not germane to Factor VIII, is there any other suggestion in Livesey that trehalose should be used in the absence of albumin as a freeze-drying cryoprotectant/stabilizer for anything at all (even a suspension) in the absence of albumin? The only discussion of potential cryoprotectants and dry stabilizers (or dry protectants) in general is in column 9, lines 16-39. Trehalose is discussed in general along with the characteristics of a number of other cryoprotectants/stabilizers. Various combinations are also discussed, including, at line 38, human serum albumin plus trehalose. There is nothing in this paragraph that suggests that it would be advantageous or desirable to use trehalose in the absence of human serum albumin for Factor VIII or that the nebulizing method required by Livesey (which is the point of the whole disclosure) would lead to the ability to use trehalose alone in the absence of serum albumin in instances where serum albumin was thought to be required.

The teaching of Livesey, taken as a whole, is mainly directed to a method for preserving "a suspension of biological material" which requires nebulizing a cryosolution *that contains the suspension* to form microdroplets which permits fast cooling at a rate which "results in the formation of amorphous phase ice and/or cubic phase ice with or without hexagonal phase ice in said cryosolution" (column 24, lines 45-48), followed by drying by molecular distillation. Any

teaching of Livesey with regard to the nature of the cryoprotectants required and the type of materials that can be dried needs to be viewed through this lens.

Furthermore, the examiner is misreading the present claim if he considers that, in the invention, the trehalose is being used as a cryoprotective agent. For example, in the middle of page 3 of the office action dated December 17, 2004 the examiner stated that:

> However, both references suggest that the claimed agent, trehalose, in the absence of albumin, is a cryoprotective agent useful in protecting proteins, such as Factor VIII, from damage during freeze-drying.

There are other references to trehalose as a cryoprotectant at the top of page 4, top of page 7 and bottom of page 8 of that office action. However, the claim under consideration reads:

> A method for preparing a stable dried composition of native Factor VIII containing a stabilising amount of trehalose in the absence of a stabilising amount of albumin.....

Hence, in the present invention, the trehalose is serving to stabilise the dried composition, rather than acting (solely) as a cryoprotectant.

In summary, even putting Curtis and Livesey together fails to make any suggestion of the invention taken as a whole. The Examiner is correct that trehalose is a known cryoprotectant. Both Curtis and Livesey verify that point. But the claim is not limited to the use of trehalose as a cryoprotectant. The claim is directed specifically to a method to freeze-dry native Factor VIII, using trehalose as a stabilizer, in the absence of albumin (thought by the art to be required) and from an aliquot of a solution. Curtis teaches nothing about freeze-drying *native* Factor VIII at all, and Livesey, to the extent that it could be said to relate to the freeze-drying of Factor VIII, teaches that this be done not from an *aliquot* of a *solution*, but rather on a frozen powder obtained by nebulizing *microdroplets (not aliquots)* of a cryosolution. Trehalose, while

mentioned as a cryoprotectant and dry stabilizer, is not the focus of either disclosure even to the extent that cryoprotectants and dry stabilizers are mentioned; other cryoprotectants and dry stabilizers are described and discussed as well. Where trehalose is used absent albumin in Example 5 of Livesey for a suspension of virus as a putative stabilizer, it appears to be non-functional.

Applicant is at a loss to see how any combination of the teaching of Curtis with the teaching of Livesey makes any suggestion that native Factor VIII be successfully freeze-dried in the presence of trehalose, and the absence of albumin, from an aliquot of a solution thereof.

The Examiner's position that Curtis teaches the lyophilization even of activated Factor VIII in the presence of trehalose but the absence of albumin is clearly erroneous; the contention that mentioning trehalose and albumin in a list of possible cryoprotectants in the alternative makes this suggestion is in error. The art considered albumin a necessary stabilizing component for Factor VIII specifically; the fact that other proteins or biological materials may not have this requirement was evidently not considered. Even assuming that this were the teaching of Curtis, it is not true, as the Examiner contends, that Livesey remedies the failure of Curtis to discuss freeze-drying of native Factor VIII by illustrating freeze-drying of native Factor VIII under the conditions specified by the claims. If this were true, there would be no need to apply the Curtis document at all. Livesey has no generic teaching of use of trehalose in any freeze-drying process in the absence of albumin; to the extent a preservation process involving trehalose is exemplified, it is only in a situation where there was no understanding in the art that albumin would be required and it is only in the context of the specialized (and distinct) molecular distillation process.

ii) There is no motivation to combine Curtis with Livesey

In view of the failure of the documents as combined to suggest the invention, it is difficult to see what motivation there would be to combine these documents even if the teachings of the invention are taken into account. It is probably unnecessary to cite support for the black-letter law that it is impermissible to use the invention as a guide to motivate the combination of documents or to pick and choose only those portions of the documents that appear to relate to the invention, ignoring the teachings of the documents taken as a whole. But see *W.L. Gore & Associates v. Garlock, Inc.*, 721 F2d 1540, 220 USPQ 303, 316 (Fed. Cir. 1983); *Orthopedic Equipment Co. v. United States*, 702 F2d 1005, 217 USPQ 193 (Fed. Cir. 1983).

As was pointed out on the record below, in order to support a rejection based on a combination of documents, some rationale must be offered by the Examiner to support this motivation (*In re Rouffet*, 47 USPQ2d 1453 (Fed. Cir. 1998)). The *Rouffet* panel recognized three possible motivations: a suggestion in the documents themselves, the commonality of the nature of the problem to be solved with that of the invention, and an extremely high profile of at least one of the documents (*e.g.*, the Kohler and Milstein paper on monoclonal antibodies with which all would be familiar). None of these appear to be present here.

In the Advisory Action, in response to applicant's assertion that no rationale for combination was provided, the Examiner provided the following.

> One of ordinary skill clearly would have derived from the disclosure of the references that the claimed preservative agent, trehalose, was well known to be suitable for preserving the claimed therapeutic agent, Factor VIII, in the absence of albumin using the claimed steps. Thus references have the use of trehalose in the preservation of Factor VIII as the common disclosure and therefore meet the *Rouffet* commonality element urged by the applicant as being missing.

First, neither document teaches that "trehalose was well known to be suitable for preserving the claimed therapeutic agent, Factor VIII, in the absence of albumin using the claimed steps." The documents simply do not teach this. If both documents suggest trehalose (among many other stabilizers) in the freeze-drying preservation of Factor VIII (as well as many other materials in the case of Livesey) this does not amount to a suggestion to combine them even if the Examiner's statement were factually accurate. And it is not factually accurate. Curtis is silent on the preservation of native Factor VIII by freeze-drying and never suggests freeze-drying anything in the absence of albumin. In Livesey, Factor VIII appears on a laundry list of biological materials that are to be prepared, using the required technique involving the formation of microdroplets and supercooling to control the nature of the formation of ice crystals, followed by drying, where trehalose is, again, simply listed on a laundry list of possible cryoprotectants. Neither document even suggests the specific combination of Factor VIII (in any form) with trehalose and freeze-drying.

In determining whether motivation exists, the documents combined need to be considered for the teaching they present as a whole. Curtis, taken as a whole, simply teaches a method to prepare the activated form of Factor VIII from native Factor VIII. One who is interested in methods of freeze-drying would not look to Curtis for any teaching in that regard. Livesey, *et al.*, at least is concerned with cryoprotection and (to an extent) freeze-drying, but the teaching of Livesey is focused on the formation of microdroplets so that these can be rapidly frozen in a manner to control the formation of ice crystals and careful manipulation of drying conditions to permit molecular distillation, not freeze-drying. It is not concerned with the efficacy of any particular cryoprotectant formulation. Livesey is directed specifically to a process that is not even included within the scope of the present claims. One seeking to find the correct conditions

for the standard procedures of freeze-drying claimed – *i.e.*, by freeze-drying aliquots of solutions, would not look to Livesey for guidance since Livesey is concerned with how to dry suspensions of biological materials in cryosolutions by nebulizing them into microdroplets, followed by rapid freezing to very low temperatures and then transferring a sample of powder into another device for molecular distillation.

Where is the suggestion in either document that it be combined with the other? Where is the commonality of the problem to be solved? And it does not appear that either document is so well known that all in the art would be aware of it.

As is clear from the passage from the office action quoted above, any motivation to combine Curtis with Livesey could only have come from advance knowledge of the disclosure of the invention itself. The claims involve Factor VIII and trehalose, so the Examiner has ignored the overall teachings of each of these documents and simply looked for these words in the texts, however incidentally they may be mentioned. In Curtis, trehalose is mentioned only once and in an entirely different context; either in freeze-drying of activated Factor VIII or in connection with native Factor VIII used as a starting material that is not being freeze-dried. In Livesey, Factor VIII appears on a laundry list of possible biological materials and trehalose is mentioned entirely independently of this laundry list on another laundry list of possible cryoprotectants. Livesey's actual exemplification in example 5 is in a context completely irrelevant to Factor VIII as there is no suggestion that serum albumin would have been required to stabilize the viral particles used in this example, whereas those in the art did consider albumin to be necessary for the stabilization of Factor VIII.

For the reasons stated above, the Examiner has failed to present an acceptable rationale for any motivation to combine Curtis with Livesey.

iii) Conclusion

The combination of Curtis with Livesey does not suggest the invention taken as a whole. Neither document addresses the problem that is solved by the invention (nor do the documents taken together) – *i.e.*, that of successfully freeze-drying native Factor VIII in the absence of a stabilizing amount of serum albumin, which (because it may contain viruses or prions) is an undesired contaminant. Curtis is concerned not with eliminating serum albumin from preservation procedures, but rather with a preparation of a different material – activated Factor VIII. Livesey is not directed to specific choices of cryoprotectants, but rather to a specific procedure, not included within the scope of the claims, for preserving biological materials which requires the nebulization of a bulk suspension of biological particles to form microdroplets, which are then frozen to form a frozen powder that must be transferred to another vessel for drying (rather than freeze-drying the aliquots of solutions of the Factor VIII required by the claim). The Livesey process is unsuitable for preparing an intravenously injectable product such as Factor VIII. The relevance of the two documents cited is seen by the Examiner not in the context of the invention taken as a whole, but only a small portion of the invention – the use of trehalose as a cryoprotectant *per se*. Clearly, that is not the invention claimed.

Further, the only basis found by the Examiner for combining the documents is that both mention Factor VIII and both mention trehalose, albeit in contexts that are irrelevant to the invention taken as a whole.

Applicant respectfully requests that the Board reverse this rejection.

B. The rejection of all claims over Curtis and Livesey as taken above in combination with Bhattacharva, *et al.*

This is not argued independently. The rejection on this basis is believed in error for the same reasons as those set forth above with regard to Curtis and Livesey taken alone.

8. Claims Appendix

Attached hereto is a copy of the claims involved in the Appeal.

9. Evidence Appendix

For the convenience of the Office, certain documents submitted as evidence below are included in the Appendix. A listing of these documents is provided on the Appendix itself.

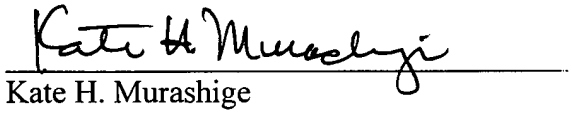
10. Related Proceedings Appendix

Not applicable.

The Assistant Commissioner is hereby authorized to charge any additional fees under 37 C.F.R. § 1.17 that may be required by this Brief, or to credit any overpayment, to **Deposit Account No. 03-1952**.

Respectfully submitted,

Dated: July 22, 2005

By: 

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CLAIMS APPENDIX

1-13. (canceled)

14. A method for preparing a stable dried composition of native Factor VIII containing a stabilizing amount of trehalose in the absence of a stabilizing amount of albumin which method comprises freeze-drying an aliquot of aqueous solution of Factor VIII containing trehalose and free of albumin.

15. The method of claim 14 wherein said native Factor VIII is plasma derived.

16. The method of claim 14 wherein said native Factor VIII is recombinant.

17-19. (canceled)

20. The method of claim 14 wherein said aqueous solution further contains L-histidine.

21. The method of claim 15 wherein said aqueous solution further contains L-histidine.

22. The method of claim 16 wherein said aqueous solution further contains L-histidine.

EVIDENCE APPENDIX

This appendix contains, for the convenience of the Office, the following evidentiary material already of record:

1. Declaration of Sam L. Helgerson regarding differences between activated Factor VIII and native Factor VIII (submitted with the Response filed 1 December 2004);

2. Declaration of E. G. D. Tuddenham, also regarding differences between activated Factor VIII and native Factor VIII (submitted with the Response filed 1 December 2004);

3. Vehar, G. A., *et al.*, *Nature* (1984) 312:337-342, comparing the structure of activated Factor VIII and native Factor VIII (submitted with the Response filed 1 December 2004);

4. Definitions of "solution" and "suspension" from a college Chemistry textbook and Stedman's Medical Dictionary (submitted with the Response filed 1 December 2004); and

5. Exhibits attached to a Response filed 25 September 2002, establishing that Factor VIII is consistently prepared in the presence of albumin for stabilization, along with pages 6-8 of the submission, which characterized the exhibits.

RELATED PROCEEDINGS APPENDIX

There are none.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Bruce Joseph ROSER

Serial No.: 09/888,734

Filing Date: 25 June 2001

For: DRIED BLOOD FACTOR COMPOSITION COMPRISING TREHALOSE

Examiner: Francisco Chandler Prats

Group Art Unit: 1651

Declaration of Sam L Helgerson

under 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Sam L Helgerson, declare as follows:

1. I am the same Sam L. Helgerson who is a co-inventor for US patent No.5,824,780 (Curtis *et al*). I have been with the Baxter Healthcare Corporation since 1991 and have worked during that time as a Research Scientist on projects to develop protein-based biotherapeutic products. I am now Senior Research Director in the Baxter BioScience R&D Group.

2. I have reviewed the Office Action mailed September 1, 2004 and I note the comments on page 6 stating that "the proteins (i.e., native Factor VIII and activated Factor VIII) possess numerous virtually identical amino acid sequences." However, although Factor VIIIa shares all of its amino acid sequence with Factor VIII, the two are in fact significantly different from one another in several key structural and functional aspects a was stated in the Background section of Patent 5,824,780: "Recent advances in the isolation of Factor VIII and the molecular cloning of the Factor VIII gene have revealed that the primary structure of Factor VIII contains several distinct types of structural domains. There are three A domains, A1, A2, and A3 each of approximately 350 amino acids, a unique region of about 980 amino acids called the B domain, and a carboxyl-terminal region of about 300 amino acids

called the C1-C2 domain. These domains are arranged in human Factor VIII in the order of A1-A2-B-A3C1-C2 (Vehar et al. Nature 312:327 to 342, 1984). Treatment of procoagulant protein Factor VIII with thrombin results in an increase in coagulant activity, which is associated with the formation of an activated form of Factor VIII. Previous attempts to isolate and characterize the activated form of human Factor VIII have been unsuccessful because the activity of this form rapidly decays. The activation of Factor VIII by thrombin has been shown to coincide with cleavage of the polypeptide chain at residue position 372 between the A1 and A2 domains, at position 740 between the A2 and B domains, at unidentified positions within the B domain, and at position 1689 between the B and A3-C1-C2 domains. The active Factor VIII complex then forms as a heterotrimer composed of the A1, A2, and A3-C1-C2 subunits." Hence, both the gross molecular sizes and the intramolecular subunit interactions of the two proteins are very different. Importantly, the activation of FVIII to FVIIIa is required in order to achieve the fully functional properties required for blood coagulation activity.

3. Page 6 of the office action also states that "at the very least, Curtis establishes generally that Factor VIII has a therapeutic utility that can be preserved upon freeze-drying in the presence of trehalose." I believe that this may overstate the utility of our work. In the Curtis *et al* patent (on which, as noted above, I was a co-inventor), we focused the disclosure to methods and formulations for stabilising the final activated Factor VIII protein, in other words Factor VIIIa. The protein structure of FVIIIa required for functional blood coagulation activity is highly dependent on specific intramolecular subunit interactions that are unique to FVIIIa in comparison to FVIII. In particular, these subunit interactions are very labile and must be stabilized in order for the desired activity to be maintained. Our work with protein stabilizing agents, i.e., human serum albumin, sucrose, and trehalose, was aimed specifically at solving this problem. We did not seek to extend the teaching of the patent disclosure to unactivated Factor VIII, in other words simply "Factor VIII". I believe that a person working in this field would have duly noted this and would not have assumed that the patent was teaching methods and formulations for stabilising Factor VIII. Because the two protein forms are so different from one another, the attributes of, uses of, and techniques involving one may not simply extrapolated to the other.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at Fremont, CA, on 30 November 2004.

(city) (state) (day)

Aam S. Helgerson

(name)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Bruce Joseph ROSER

Serial No.: 09/888,734

Filing Date: 25 June 2001

For: DRIED BLOOD FACTOR COMPOSITION COMPRISING TREHALOSE

Examiner: Francisco Chandler Prats

Group Art Unit: 1651

DECLARATION OF EDWARD G. D. TUDDENHAM

UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Edward G D Tuddenham, declare as follows:

1. I am Professor of Haemostasis at the Imperial College Faculty of Medicine, London, UK. I am familiar with the terminology used in the field of blood-derived factors, and with the nature of Factor VIII. I have been practicing in this field for thirty years. A copy of my *curriculum vitae* is attached.

2. I have reviewed the above-referenced application in its entirety. This application nowhere describes the treatment of Factor VIII with thrombin or any other protease to effect its activation. At no location is "activated Factor VIII" referred to. If such treatment had been performed, the "Factor VIII" would be referred to as "activated Factor VIII" or some additional

description would be given to indicate that the Factor VIII as it would be found in plasma, or as it would be recombinantly produced using the gene encoding that found in plasma, had been treated with a protease. I have been told that the record in this application indicates that the US Patent Office ("the Office) agrees with the foregoing, and that the Office understands the claims as presently presented are directed to methods to prepare a stable, dried composition of "native" Factor VIII, i.e., they refer to a Factor VIII other than that "activated" by thrombin treatment.

3. I have reviewed the Office action mailed 1 September 2004 and note the comments on page 6 stating that "the proteins (i.e., native Factor VIII and activated Factor VIII) possess numerous virtually identical amino acid sequences." However, substantial portions of the native form are missing from activated Factor VIII. Factor VIII is a heterodimer of a heterogeneous 90 - 210 kD heavy chain, having a 90 kD constant region and a variable region of up to 120 kD, and a light chain of 80 kD. This is the case whether the Factor VIII is in the form that circulates in the plasma, or the commercial form obtained from plasma, or the commercial form obtained by recombinant DNA techniques. However, *activated* Factor VIII is a hetero*trimer* containing only a portion of the native Factor VIII light chain (73 kD) and two fragments of the constant region of the heavy chain of 50 kD and 43 kD. Although some amino acid sequences are retained, they are rearranged and a very large portion (amounting to an average of 124 kD (out of a total heterodimeric mass of 290 kD)) is missing. Thus, almost half of the amino acid sequences of native Factor VIII are missing from activated Factor VIII.

4. Page 6 also states "at the very least, Curtis establishes generally that Factor VIII has a therapeutic utility that can be preserved upon freeze-drying in the presence of trehalose." This is not entirely accurate. Curtis concerns only preservation of activated Factor VIII, which activated Factor VIII does have therapeutic activity, while native Factor VIII does not (absent

activation by protease). The behavior of the already active molecule is not informative with respect to the behavior of the circulating heterodimeric form, which represents native Factor VIII, since that form is not active at all. Indeed it is referred to in the literature as the 'procofactor', which is analogous to the zymogen of the protease factor thrombin called prothrombin. Thus, even if Curtis showed that the activated form of Factor VIII, which itself has cofactor activity, could be stably preserved in the presence of trehalose, there is no scientific basis to extrapolate this to the native Factor VIII, which lacks such cofactor activity until proteolytically cleaved by thrombin. It simply does not follow. Great care is taken in the literature and in practice to distinguish between the inactive pro- forms of clotting factors and their activated forms as these have highly different properties. For example the activated forms are thrombogenic and have shortened half-lives *in vivo*.

5. In my opinion, the activated form of Factor VIII and the native form of Factor VIII (as a circulating heterodimer) are sufficiently different that the physical behavior of one is not predictive of the physical behavior of the other. Not only is almost half of the amino acid sequence missing in the activated form; the arrangement of the remaining peptides is different. Instead of a heterodimer with a heterogeneous heavy chain of 90-210 kD and a light chain of 80 kD, the activated form is a trimer that is not heterogeneous and comprises two segments of 50 and 43 kD, as well as a 73 kD monomer. In view of the heterogeneity of the native Factor VIII and in view of its markedly different structure from activated Factor VIII, the behavior of these materials would be expected to be very different rather than similar.

6. I have also reviewed U.S. Patent 5,364,756 to Livesey, *et al.* I have noted that claim 17, dependent on claim 1, specifies the material subjected to the process of claim 1 as Factor VIII. Claim 1 is directed to a method for preserving a suspension of biological material,

which comprises preparing a cryosolution using a suspension of biological material. With respect I submit that to chemists, pharmacologists and biologists a suspension means particulate matter held dispersed in some fluid, not a molecular substance dissolved in some aqueous solution. I am familiar with the solubility of characteristics of Factor VIII and can verify that a suspension of Factor VIII as condensed perhaps crystalline particles could reasonably be prepared only by use of extremely high concentrations that are not realistically contemplated or by denaturing the protein. The suspensions exemplified in the Livesey patent are of insoluble materials such as cells or viruses. The description of Livesey is inappropriate to Factor VIII, which is alluded to, inexplicably, in a 'shopping list' of materials that reasonably relate to Livesey's process in column 4, lines 57-64. A skilled practitioner of the art, familiar with the characteristics of Factor VIII, would understand that the inclusion of Factor VIII in such a list in this context is clearly an error or an optimistic attempt to be all encompassing and hence over inclusive.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at London, United Kingdom, on 23rd November 2004.
(city) (state) (day)

E. G. D. Tuddenham
(name)

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Structure of human factor VIII

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The deduced amino acid sequence of human factor VIII, obtained from the DNA sequence, predicts a mature polypeptide of 2,332 amino acids containing a triplicated domain structure. The polypeptide has 35% sequence homology with the copper-binding plasma protein, ceruloplasmin. Determination of the thrombin cleavage sites in plasma-derived factor VIII polypeptides allows prediction of the domains involved in the associated activation and inactivation of the protein.

PREPARATIONS of factor VIII/von Willebrand factor complex[1-12] contain four closely related properties or activities: factor VIII coagulant activity, an antigen associated with the factor VIII coagulant activity, platelet adhesion promoting activity and an antigen precipitated by antisera raised against the purified complex (factor VIII-related protein). Factor VIII separated from the complex has associated trace amounts of protein, is unstable and consists of multiple polypeptide chains[13-17], hindering detailed characterization studies.

The purification of human factor VIII by affinity to a monoclonal antibody directed against the coagulant activity of factor VIII[18], has allowed characterization of the protein fragments of factor VIII or thrombin-activated factor VIII by partial amino acid sequence analysis. This sequence information has been used to isolate cDNA and genomic clones encoding human factor VIII[19,20]. The protein sequence deduced from these clones, together with an analysis of the cleavage products associated with the activation of factor VIII by thrombin, allows the assignment of thrombin cleavage sites and the identification of most of the polypeptide fragments present in highly purified factor VIII preparations. The factor VIII sequence exhibits striking homology with the plasma copper-binding protein ceruloplasmin, suggesting novel biochemical activities for factor VIII as well as a role for metal ions other than calcium in the blood coagulation cascade.

Analysis of plasma factor VIII

Preparations of human factor VIII[18] purified over 300,000-fold from plasma contained several proteins of relative molecular mass (M_r) 210,000–80,000 (Fig. 1*A*). These protein bands were not connected by disulphide links because samples analysed under non-reducing conditions gave a similar pattern (data not shown). To determine the relationship of these multiple polypeptide chains, we analysed them by tryptic peptide mapping. The preparation used in Fig. 1*B* contained a protein of M_r 240,000, producing a peptide map which did not show identity with the other proteins of the mixture (Fig. 1*B*, *a*), and has been found to be a von Willebrand factor subunit (data not shown). Peptides of M_r 90,000–210,000 all had a common tryptic map, indicating that they are derived from the same or closely related polypeptide chains (Fig. 1*B*, *b–f*). Furthermore, Western blot analysis of the factor VIII preparations demonstrated that a factor VIII-specific monoclonal antibody[18] reacted with the M_r 90,000–210,000 polypeptides (data not shown). Two very similar patterns generated by the proteins of M_r 80,000 and 70,000 had a different peptide map (Fig. 1*B*, *g*, *h*). These results demonstrate that the fragments of M_r 90,000–210,000 are structurally related and could be pooled and treated as one polypeptide chain. The protein represented by the band of M_r 80,000 (and 70,000 when present) was analysed separately.

The purified factor VIII preparations were fractionated by gel filtation on a TSK 4000SW HPLC column; analysis of the resulting fractions demonstrated the effective separation of M_r 240,000 protein, the polypeptides of M_r 90,000–210,000 and the M_r 80,000 fragment (Fig. 2*B*). We performed amino acid sequence analysis on peptides generated from the M_r 80,000 protein, the polypeptide pool of M_r 90,000–210,000 and a fragment of M_r 90,000 from limited thrombin digestion of the M_r 90,000–210,000 pool. After digestion of each sample with trypsin, the resulting peptides were separated by reverse-phase HPLC and sequenced. The peptide sequence AWAYFSDVDLEK, used to prepare synthetic DNA probes identifying factor VIII genomic DNA clones, is indicated in Fig. 2*C*.

Structure of factor VIII protein

The molecular cloning of the entire factor VIII coding region is described in an accompanying paper[19]. The 2,351-amino acid sequence for factor VIII, deduced from the nucleotide sequence of these clones, is shown in Fig. 3. The first 19 amino acids of the sequence comprise the signal sequence for factor VIII, based on peptide sequence analysis of a fragment derived from the M_r 90,000–210,000 polypeptide pool. The N-terminal sequence of this M_r 30,000 fragment, obtained as a thrombin digest product of the M_r 90,000–210,000 pool, is identical to the first 12 amino acids which follow the predicted factor VIII leader sequence (see Fig. 5). This presequence exhibits a core of 10 hydrophobic amino acids flanked by two charged residues, a structure which conforms to that observed for the leader sequences found in most secreted proteins[21]. The mature protein contains 2,332 amino acids (calculated M_r 264,763).

The availability of the complete factor VIII sequence reveals the organization and identity of the tryptic peptides obtained from the pools of separated plasma-derived factor VIII fragments. Essentially all tryptic peptide sequences determined from the M_r 90,000–210,000 protein pools are located in the amino-

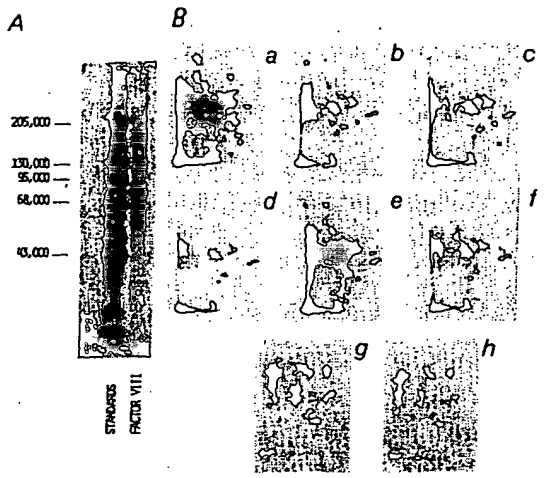

Fig. 1 *A*, Purified human factor VIII analysed by SDS-polyacrylamide gel electrophoresis. *B*, Two-dimensional tryptic mapping of factor VIII polypeptide chains. The resulting tryptic patterns of proteins of M_r: *a*, 240,000; *b*, 210,000; *c*, 170,000; *d*, 150,000; *e*, 120,000; *f*, 100,000; *g*, 80,000; and *h*, 70,000 are shown.
Methods: The purified protein[18] was reduced and analysed in a 5–10% polyacrylamide gradient gel in the presence of SDS by the procedure of Laemmli[37]. The molecular weights of the protein standards are shown (myosin, β-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin). The bands were detected using the silver stain procedure of Morrissey[38]. 1 μg of a factor VIII preparation was denatured in 1% SDS and labelled with 300 μCi ^{125}I for 15 min using the iodobead procedure[38]. Labelled polypeptides were located on dried SDS-polyacrylamide gels by autoradiography and digested by incubation of gel slices with 10 μg trypsin in 0.1 M ammonium bicarbonate buffer for 6 h at 37°C. After repeated lyophilizations, peptides were dissolved in 8.8% formic acid and a portion was subjected to thin-layer electrophoresis in the same buffer (400 V for 45 min) on pre-coated TLC-cellulose plates (E. Merck, Darmstadt, FRG). For the second dimension, peptides were separated by ascending chromatography in *n*-butanol/pyridine/glacial acetic acid/water, 75:50:15:60 (v/v). The plates were then subjected to autoradiography.

terminal half of the molecule, whereas those sequences obtained for the M_r 80,000 fragment are found at the carboxy-terminus of the factor VIII sequence (unpublished results). The most carboxy-terminal tryptic peptides identified for the M_r 90,000–210,000 pool gave the sequences GEFT and –QEE, beginning at positions 1,155 and 1,194, respectively. This shows that the M_r 210,000 fragment consists of a protein of $M_r \geq 135{,}000$ containing 14 potential asparagine-linked glycosylation sites. The location of the M_r 80,000 fragment of factor VIII is delineated by two peptide sequences which define a stretch of ~680 amino acids. The first of these was obtained from the amino-terminal sequence of the M_r 80,000 fragment beginning at position 1,649 (see Fig. 5), whereas the second corresponds to a tryptic peptide, MEVLGCEAQDL, 12 amino acids from the C-terminus predicted by the DNA sequence. Thus, there is no significant removal of C-terminal sequences from the plasma-derived molecule. The failure to recover tryptic peptide sequences from the region between position 1,200 and the M_r 80,000 fragment is probably due to the relatively low concentration of the M_r 210,000 species in the M_r 90,000–210,000 fragment pool. This position should therefore be considered the minimal C-terminal extent of the M_r 210,000 protein.

Computer-aided analysis of the factor VIII protein sequence revealed two types of internal homology: the first consists of a triplicated segment (A domain) found at positions 1–329, 380–711 and 1,649–2,019 of the mature polypeptide (Fig. 4); the second and third domains of the triplication are separated by a region of 983 amino acids (B domain) extremely rich in potential asparagine-linked glycosylation sites. In addition, an unrelated duplication of 150 amino acids is found at the C-terminus of the molecule (C domain). The A domains have ~30% amino acid homology, whereas the C domains are ~40% homologous. Most

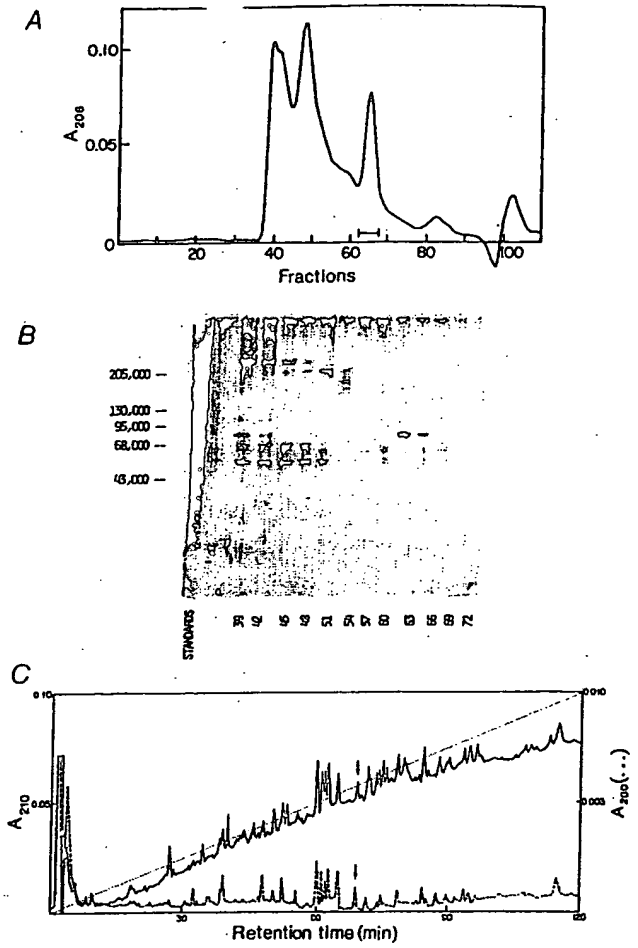

Fig. 2 Purification of factor VIII polypeptides. *A*, Fractionation of proteins by TSK 4000 HPLC. *B*, SDS-polyacrylamide gel analysis of fractions from TSK 4000 chromatography. *C*, Reverse-phase HPLC separation of tryptic peptides of M_r 80,000 protein.
Methods: *A*, Human factor VIII preparations[18] were dialysed into 1% ammonium bicarbonate containing 0.1% SDS. The samples were lyophilized and stored at −20°C until use. The samples were reconstituted in distilled water and applied to a TSK 4000 SW column (0.75×50 cm; Alltech Associates, Deerfield, Illinois) equilibrated with 0.1 M sodium phosphate buffer, *p*H 7.0, containing 0.1% SDS. Samples of ~0.15–0.25 ml were injected and the column developed isocratically at a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at either 206 nm or 280 nm and fractions (0.2 ml) collected. Fractions across the profile were reduced and analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 1 legend. The protein of M_r 80,000 was pooled for tryptic digestion as shown in *A*. *C*, The TSK 4000 SW purified protein of M_r 80,000 (0.8 nM) was dialysed under a nitrogen atmosphere overnight against 0.36 M Tris-HCl, *p*H 8.6, containing 8 M urea, 3.3 mM EDTA and 10 mM dithiothreitol, DTT (final vol. 1.5 ml). The protein was alkylated by adding 15 μl of 5 M iodoacetic acid (dissolved in 1 M NaOH). The reaction was allowed to proceed for 35 min at room temperature in the dark and was quenched by adding DTT to a final concentration of 100 mM. The protein solution was dialysed against 8 M urea in 0.1 M ammonium bicarbonate for 4 h. The urea dialysis solution was changed over a period of 24 h to gradually reduce the urea concentration to a final level of 0.5 M. Trypsin was added at a weight ratio of 1:50 at 37°C for 12 h. HPLC separation of the resulting tryptic peptides was performed on a high-resolution Synchropak RP-P C-18 column (0.46×25 cm; 10 μ). The column was developed with a gradient of acetonitrile (1–70% in 200 min) in 0.1% trifluoroacetic acid. Absorbance was monitored at 210 nm and 280 nm. Each peak was collected and stored at 4°C until subjected to sequence analysis in a Beckman spinning cup sequencer with on-line phenylthiohydantoin amino acid identification[40]. The arrow identifies the peptide (AWAYFSDVDLEK) resulting in identification of a factor VIII genomic clone[19,20].

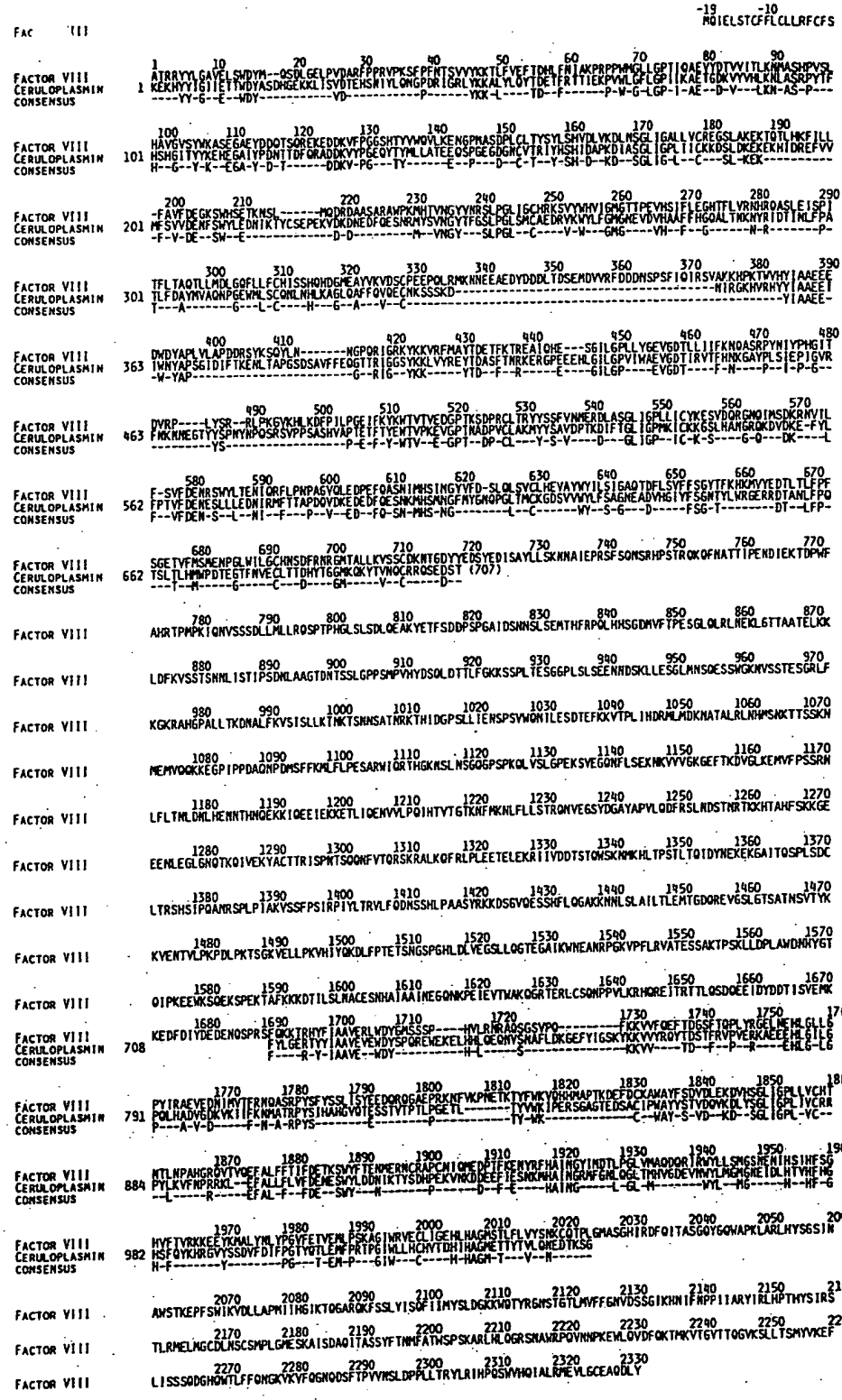

Fig. 3 Homology of human factor VIII with human ceruloplasmin. The deduced factor VIII amino acid sequence derived from cDNA and genomic clones[19,20] is compared with that of human ceruloplasmin[22]. The consensus line shows residues which are identical in the two proteins. The numbering above the sequences is that of factor VIII. The number preceding the ceruloplasmin line represents the numbering of the amino acid sequence beginning the line. The single-letter notation for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

of the 23 cysteine residues of the mature polypeptide are clustered in the A and C domains and occupy similar positions Fig. 6), suggesting that the structures of both repeated domains of factor VIII reflect conserved disulphide bonding arrangements.

The A domains of the factor VIII protein show striking homology with the copper-binding plasma protein ceruloplasmin (Fig. 3). Amino acid sequence analysis of ceruloplasmin has revealed a structure consisting of three contiguous domains sharing ~30% homology[22-24]. The triplicated domains of factor VIII and ceruloplasmin exhibit a pairwise homology of 30% (Fig. 4). Although the B domain has no substantial homology with any known sequence, the C domain shares 20% amino acid homology with the discoidin lectins from *Dictyostelium*[25].

Ceruloplasmin contains six copper atoms in three distinct types of coordination: two of type 1, one of type 2 and three electron paramagnetic resonance-nondetectable type 3 copper ions[26]. The type 1 copper ions are thought to bind to the carboxy-terminal portion of the domains of ceruloplasmin (domain residues 240-350; Fig. 4) based on sequence homology with the type 1 copper-binding protein plastocyanin[27]. The four amino acid side chains proposed as the ligands for the type 1

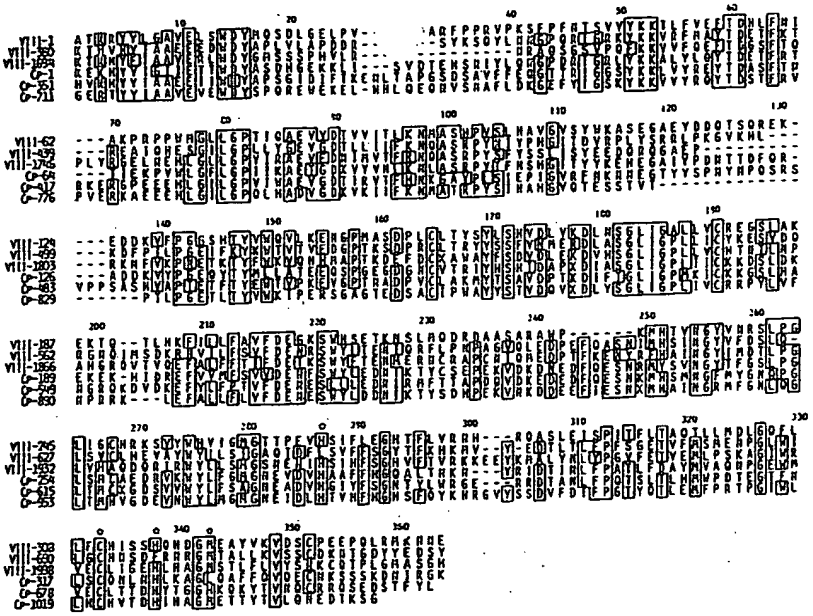

Fig. 4 Domain homology of human factor VIII and human ceruloplasmin[22]. Identical residues in four of the six domains are boxed. The protein is indicated to the left of the lines (VIII, factor VIII; Cp, ceruloplasmin) with the associated number indicating the position in the sequence of the first amino acid of the line. A domain residue numbering system is shown above the sequences. * Residues believed to be the ligands for type 1 copper (based on homology to plastocyanin[27]).

copper atoms of ceruloplasmin are found in the first and third A domains of factor VIII (histidines 287 and 338, cysteine 333 and methionine 343; see Fig. 4). The conservation of the copper-ligand residues found in ceruloplasmin strongly suggests similar metal binding characteristics for factor VIII.

Thrombin cleavage

The coagulant activity of factor VIII is increased markedly by treatment with catalytic amounts of the serine protease thrombin[28]. Thrombin activation of factor VIII is associated with a series of polypeptide cleavages[13–17]. Further incubation with thrombin leads to degradation of the protein with a concomitant loss of coagulant activity[13–17]. To understand the structural basis for these observations, the separated pools of factor VIII fragments were subjected to thrombin digestion and the resulting products characterized by SDS-polyacrylamide gel electrophoresis. Thrombin digestion of the M_r 80,000 protein resulted in a product of M_r 73,000 (Fig. 5*A*). Treatment of the polypeptide pool of M_r 90,000–210,000 led initially to the appearance of two bands of M_r 43,000 and 50,000 (Fig. 5*A*). Longer incubation with thrombin resulted in the conversion of the M_r 50,000 fragment to polypeptides of M_r 30,000 and 20,000 (data not shown). The M_r values of these thrombin digest fragments are similar to those generated by thrombin treatment of native factor VIII preparations[13,15,16]. Amino-terminal sequence analysis was performed on the separated protein chains before and after thrombin digestion. The resulting sequences are compared with the corresponding amino acid sequences deduced from the factor VIII cDNA sequence[19] in Fig. 5*B*; also shown are the potential cleavage sites found at the amino-terminus of the M_r 80,000 protein and that which separates the M_r 90,000 fragment from the carboxy-terminal portion of the M_r 210,000 protein. The sequence surrounding the latter potential cleavage site (position 740) is similar to the amino-terminal sequence of the M_r 70,000 protein (-PRSF---RH-) (Fig. 5*B*). There is no other consensus sequence that would predict the specificity of thrombin cleavage. A homologous stretch preceeding the thrombin cleavage sites for the M_r 43,000 and 73,000 proteins is observed (Fig 5*B*), but whether this homology determines thrombin specificity or simply reflects the internal duplication is uncertain. The most consistent sequence found at thrombin cleavage sites within factor VIII is an arginine residue followed by either serine or alanine. Other such sequences (-RS- or -RA-) do occur within the protein but are not cleaved, suggesting the possible involvement of secondary structure in thrombin specificity. The cleavage that frees the M_r 80,000 protein occurs at an arginine-glutamic acid sequence, probably not a thrombin-generated cleavage site. This cleavage occurs quickly and is complete within the time required for isolation of the protein. The precursor factor VIII protein therefore may be cleaved to free the M_r 80,000 polypeptide by a protease other than thrombin.

Discussion

The structure of factor VIII revealed by the amino acid sequence predicted from the cloned cDNA and the structural characterization of polypeptide fragments described here are summarized in Fig. 6. The size of the factor VIII precursor moiety is consistent with the reported isolation of single-chain M_r 330,000 protein from plasma[18] and supports the notion that the protein circulates as a high-molecular weight form that is readily cleaved in plasma and/or during isolation to a series of degradation products.

The primary structure of factor VIII exhibits three distinct types of structural domain, including a triplicated region of ~330 amino acids (A domains), a unique region of 980 amino acids (the B domain) and a carboxy-terminal duplicated region of 150 amino acids (C domains), which are arranged in the order A1–A2–B–A3–C1–C2 (Fig. 6). The A domains of factor VIII show significant homology to ceruloplasmin, consisting also of a triplicated structure of three A domains but lacking both B and C domains[22–24]. Particularly striking is the clustering of cysteine residues at similar locations within related structural domains of factor VIII (Fig. 6). The determination of disulphide pairings for ceruloplasmin[23,29] predicts two types of internal disulphide bonding arrangements for the A domains of factor VIII. The disulphide structure proposed for the C domains of factor VIII is based on the proposition that the disulphide linkgages form between the two cysteine residues found in each domain. The large B domain which separates the second and third A domains of factor VIII contains only four cysteine residues, but the presence of 19 asparagine-linked glycosylation sites suggests that this region is extensively modified by carbohydrate addition.

The activation of factor X by factor IX_a in conjunction with factor VIII is known to require calcium ions. Factor IX_a and factor X both contain γ-carboxyglutamic acid residues which are thought to be involved in calcium binding. The protein-bound calcium ions mediate the interaction of these proteins with the phospholipid surface. The homology of factor VIII with ceruloplasmin suggests the possible involvement of copper or other metal ions in the role of factor VIII in factor X activation. One possibility for the role of such metal ion involvement is suggested by the binding of lanthanide ions by γ-carboxyglutamic acid residues[30]. It is interesting to speculate that the potential copper-binding ligands of factor VIII interact with a metal ion jointly bound by the γ-carboxyglutamate

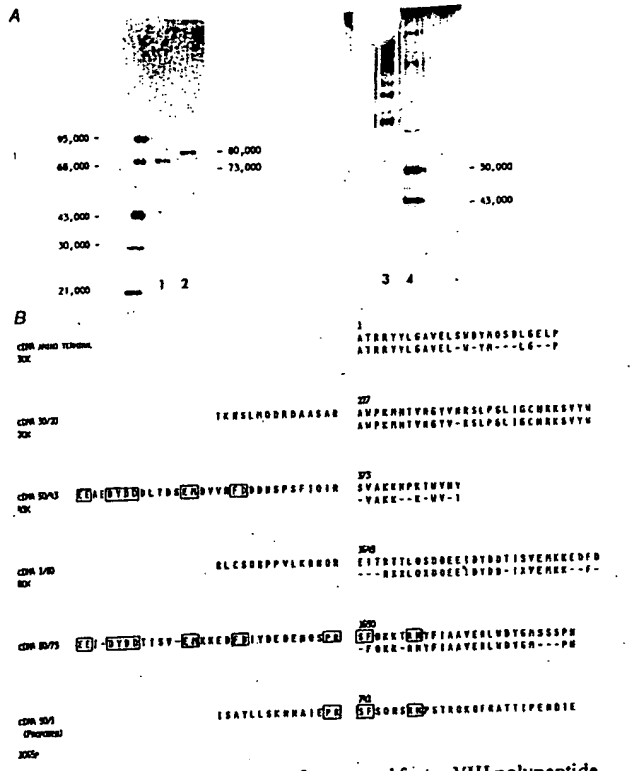

Fig. 5 *A*, Thrombin cleavage of separated factor VIII polypeptide fractions. *B*, Alignment of thrombin-generated amino termini with the deduced amino acid sequence[19].

Methods: *A*, The factor VIII polypeptides were separated as described in legend to Fig. 2*A*. SDS was removed by dialysis of the fractions against 8 M urea solutions and urea removed by dialysis against 0.01 M Tris, *p*H 8.0. Thrombin was then added at weight ratios to a maximum of 1:20. The digestions were allowed to proceed at room temperature and the extent of cleavage was monitored by SDS-gel electrophoresis. Lanes 1 and 2 are the M_r 80,000 protein with and without thrombin, respectively; lanes 3 and 4 are the M_r 90,000–210,000 protein without and with thrombin, respectively. *B*, Thrombin digestion products were separated by preparative SDS-gel electrophoresis, electroeluted by the procedure of Hunkapiller *et al.*[41] and analysed on a Beckman spinning cup sequencer[40]. The M_r 80,000 protein was obtained by TSK separation as described for Fig. 2*A*. The thrombin cleavage site is indicated by a space in the cDNA deduced sequence. The number above the subsequent amino acid corresponds to the position of that residue in the linear sequence. The amino-terminal protein sequences for the various polypeptide chains are aligned under the translated gene sequence. –, Positions where no residue could be identified; X, positions where the wrong amino acid was determined. The relative molecular masses of the proteins separated are listed (30K is the amino terminus obtained for the gel-eluted polypeptide with a M_r of 30,000); solidi indicate cleavage products (for example, 50/43 indicates the cleavage which separates the M_r 50,000 and 43,000 species). Regions which share sequence homology are boxed.

residues of factors IX or X. In addition to copper transport and haemostatis, several enzymatic functions have been ascribed to ceruloplasmin, including ferroxidase activity, amino oxidase activity and superoxide dismutase activity[31–33]. It will be important to determine whether any of these activities are associated with factor VIII.

The amino-terminal sequence of factor VIII thrombin fragments and the homology of factor VIII with ceruloplasmin provide insight into a functional purpose for the cleavages. Factor VIII isolated from plasma is usually degraded. In certain preparations, small amounts of a M_r 330,000 protein were observed when analysed on SDS-polyacrylamide gels run under non-reducing conditions; this protein was not observed when the factor VIII samples were reduced before electrophoresis. This reducible 330,000 may be a disulphide-linked, limited proteolytic degradation product of a single-chain M_r 330,000 factor VIII molecule first reported by Tuddenham *et al.*[18]. The portion of the protein immediately prior to the M_r 80,000 protein may thus be a disulphide linker region which holds the M_r 210,000 and 80,000 regions (or larger precursors) in the reducible 330,000 form. Exposure of plasma-derived protein preparations to thrombin results in activation of the factor VIII coagulant activity and the appearance of the band at M_r 90,000 (residues 1–740) due to the removal of the B domain or its fragments. Thrombin further cleaves both the M_r 80,000 and 90,000 proteins. The M_r 80,000 protein is cleaved after residue 1,689, releasing a M_r 4,500 peptide containing one potential asparagine glycosylation site. This peptide is highly acidic, containing 15 aspartic and glutamic acid residues and only 4 lysine or arginine residues out of a total of 41 amino acids. Thrombin cleavage of the M_r 90,000 protein to M_r 50,000 and 43,000 products occurs between the first two A domains of factor VIII after an acidic spacer region (15 aspartic and glutamic acid residues; 4 lysine/arginine; total of ~42 amino acids) having some sequence homology with the acidic peptide cleaved from the M_r 80,000 protein (Fig. 5*B*). Further cleavage of the M_r 50,000 protein (domain residue 240, Fig. 4) occurs immediately preceding the region having sequence homology with plastocyanin[27] thereby freeing this potential metal-binding domain.

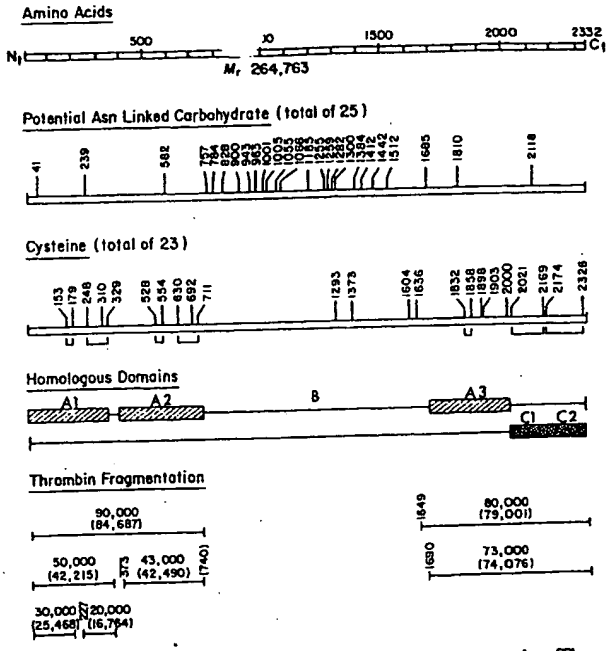

Fig. 6 Line diagram of factor VIII precursor protein. The molecular masses of the various fragments as determined by SDS-polyacrylamide gel electrophoresis are indicated (the M_r calculated from the actual amino acid composition[19] is given in parentheses). The positions of potential asparagine-linked glycosylation sites and cysteine residues are shown. The positions of the thrombin fragments and residue numbers of amino-terminal amino acids are also given. The position of cleavage of the M_r 90,000 protein has not been determined and is suggested. Boxes, the triplicated domains homologous to ceruloplasmin as well as the duplicated domain.

Factor VIII has many properties in common with coagulant protein factor V[34–36]. These proteins function in the intrinsic coagulation cascade with an activated vitamin K-dependent coagulation protein (factors IX_a and X_a for factors VIII and V, respectively), a phospholipid surface and calcium ions. These complexes result in the specific activation of a second vitamin K-dependent coagulation protein (factor X and prothrombin for factors VIII and V, respectively). Factor VIII and factor V are both proteins of $M_r > 300{,}000$; they are cleaved by a series of thrombin-catalysed events to generate proteins of M_r 90,000 (from the amino termini) and M_r 80,000 (from the carboxy

termini) from a single-chain circulating form. By analogy with factor V, the M_r 90,000 and 80,000 proteins would correspond to fragments D and E of factor V, respectively[34]. These two fragments of factor V can be separated from the activation peptides and isolated as a functional two-subunit protein[35,36]. Both subunits are required for factor V activity[35] and both may be required for factor VIII activity. A highly glycosylated intermediate region is cleaved from both proteins. Therefore, both factors V and VIII seem to be highly similar in structure, thrombin cleavage pattern and, presumably, function.

The studies described here provide a structural basis for defining the role of the diverse molecular forms of factor VIII in their interaction with other proteins of the coagulation cascade. The availability of complete factor VIII cDNA clones capable of programm. recombinant factor VIII synthesis in mammalian cell cultures[19] will offer a unique opportunity to perform similar studies with the single-chain precursor molecule. The questions raised here concerning the relationship of processing events, structural domains and homology to ceruloplasmin with the biological function of factor VIII may be answered by studying structural changes introduced into the protein by modification of these cloned DNA sequences.

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Molecular cloning of a cDNA encoding human antihaemophilic factor

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A complete copy of the mRNA sequences encoding human coagulation factor VIII:C has been cloned and expressed. The DNA sequence predicts a single chain precursor of 2,351 amino acids with a relative molecular mass (M_r) *267,039. The protein has an obvious domain structure, contains sequence repeats and is structurally related to factor V and ceruloplasmin.*

HAEMOPHILIA A is a bleeding disorder caused by deficiency or abnormality of a particular clotting protein, factor VIII:C[1] occurring in about 10–20 males in every 100,000. Afflicted individuals suffer episodes of uncontrolled bleeding and are treated currently with concentrates rich in factor VIII:C derived from human plasma. The available therapy, although reasonably effective, is very costly and is associated with a finite risk of infections. We report here significant progress in the use of recombinant DNA technology to provide pure human factor VIII:C as an alternative treatment for haemophiliacs.

Blood clotting begins with injury to a blood vessel. The damaged vessel wall causes adherence and accumulation of platelets activating the plasma proteins which initiate the coagulation process. Sequential activation, via specific proteolytic cleavages and conformational changes, of a series of proteins comprising the coagulation cascade eventually leads to deposition of insoluble fibrin which, together with aggregated platelets, curtails the escape of blood through the damaged vessel wall. Factor VIII:C is a large plasma glycoprotein that functions in the blood coagulation cascade as the cofactor for the factor IXa-dependent activation of factor X. It can be activated proteolytically by a variety of coagulation enzymes including thrombin[2].

In order to provide factor VIII:C for treatment of haemophiliacs we cloned a full-length cDNA. A major obstacle to the cloning effort was the large size of the protein, estimated to be at least M_r 250,000. Purification of factor VIII:C from plasma[3] is made difficult by its low abundance, its extreme sensitivity to degradation by serum proteases and its tight association with polymeric forms of the more abundant protein, von Willebrand factor. Fass *et al.*[4] have described a purification procedure for porcine factor VIII:C using monoclonal antibody

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EXHIBIT A

two volatile liquids is greater than that calculated from Raoult's law, the deviation is said to be positive, whereas when each vapor pressure is lower than the calculated value, the deviation is negative.

When deviation from Raoult's law is sufficiently extensive, there may be a range of concentrations in which the total vapor pressure of the solution is even higher than that of the more volatile component in the case of positive deviation, or even lower than that of the less volatile component in the case of negative deviation. In any such case there will be one particular solution that exhibits, respectively, a maximum or a minimum vapor pressure at a given temperature. The higher the vapor pressure of a particular solution at a given temperature, the lower will be its boiling point. Hence, when there are large positive deviations from Raoult's law, we may have a solution of minimum boiling point; with large negative deviations, we may have a solution of maximum boiling point.

The vapor from a solution of either minimum or maximum boiling point is of exactly the same composition as the solution itself. Hence, in these special cases, neither the composition nor the boiling point of the solution changes during distillation; on this account, such mixtures are called *constant boiling mixtures.* Alcohol and water, for example, form a minimum boiling mixture containing 95.5% of alcohol; nitric acid and water, a maximum boiling mixture containing 68% of nitric acid. Since constant boiling mixtures distill without change in composition, it is impossible in such cases to obtain both pure components from any solution by the process of fractional distillation.

THE COLLOIDAL STATE

15.22 Definitions

When a finely divided insoluble substance such as sand is shaken with water, a mixture is obtained that appears cloudy or turbid. On standing, however, the mixture soon becomes clear as the particles of sand settle to the bottom of the vessel. Such a mixture is called a *suspension.* On the other hand, if sugar or salt is stirred with water, the particles of solid completely disappear so that they cannot be observed even with the aid of the most powerful microscope; i.e., they go into solution.

Now, when powdered starch is treated with boiling water, or when arsenic(III) sulfide is formed by the passage of hydrogen sulfide through an aqueous solution of arsenic(III) oxide, a mixture is obtained that is not homogeneous, yet which shows scarcely any turbidity. The particles of insoluble solid (starch or arsenic(III) sulfide) do not settle out but remain in suspension indefinitely. Such a system, whose properties are intermediate between those of an ordinary suspension and those of a true solution, is called a *colloidal dispersion;* the finely divided substance is referred to as the *dispersed phase,* and the liquid is called the *dispersion medium.*

Although the term "colloid" (from the Greek word *kolla*—glue) was originally applied to a group of substances that readily form dispersions

Exhibit A

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Exhibit A

manipulation. 2. The performance or procedures of an operation. [L. *chirurgia;* G [illegible]*r,* hand, + *ergon,* work]

ambulatory s., o[illegible]ative procedures performed on patients who are admitted to and discharged from a hospital on the same day.

aseptic s., the performance of an operation with sterilized hands, instruments, etc., and utilizing precautions against the introduction of infectious microorganisms from without.

closed s., s. without incision into skin, *e.g.,* reduction of a fracture or dislocation.

cosmetic s., s. in which the principal purpose is to improve the appearance, usually with the connotation that the improvement sought is beyond the normal appearance, and its acceptable variations, for the age and the ethnic origin of the patient. SYN esthetic s.

craniofacial s., simultaneous s. on the cranium and facial bones.

esthetic s., SYN cosmetic s.

featural s., rarely used term for plastic s. of the face, for correction or improvement of appearance.

keratorefractive s., SYN refractive *keratoplasty.*

laparoscopic s., operative procedure performed using minimally invasive surgical technique for exposure that avoids traditional incision. Visualization is achieved using a fiber optic instrument, usually attached to a video camera.

laparoscopically assisted s., operative procedure performed using combined laparoscopic and open techniques; most commonly applied to colon or small intestinal resections with anastomosis.

major s., SEE major *operation.*

microscopically controlled s., SYN Mohs' *chemosurgery.*

minimally invasive s., operative procedure performed in a manner derived to result in the smallest possible incision or no incision at all; includes laparoscopic, laparoscopically assisted, thoracoscopic, and endoscopic surgical procedures.

minor s., SEE minor *operation.*

Mohs' s., SYN Mohs' *chemosurgery.*

Mohs' micrographic s., SYN Mohs' *chemosurgery.*

open heart s., operative procedure(s) performed on or within the exposed heart, usually with cardiopulmonary bypass (as opposed to closed heart surgery).

oral s., the branch of dentistry concerned with the diagnosis and surgical and adjunctive treatment of diseases, injuries, and deformities of the oral and maxillofacial region.

orthognathic s., SYN surgical *orthodontics.*

orthopaedic s., the branch of s. that embraces the treatment of acute and chronic disorders of the musculoskeletal system, including injuries, diseases, dysfunction and deformities (orig. deformities in children) in the extremities and spine. SEE ALSO orthopaedics.

plastic s., the surgical specialty or procedure concerned with the restoration, construction, reconstruction, or improvement in the shape and appearance of body structures that are missing, defective, damaged, or misshapen.

reconstructive s., SEE plastic s.

stereotactic s., SYN stereotaxy.

thoracoscopic s., s. done using one or more endoscopic instruments.

transsexual s., procedures designed to alter a patient's external sexual characteristics so that they resemble those of the other sex.

video-assisted thoracic s. (VATS), a less morbid alternative to "open" thoracotomy that employs cameras, optic systems, percutaneous stapling devices, and assorted endoscopic graspers, retractors, and forceps. Also called video thoracoscopic surgery, it can be selectively applied to various pulmonary, pleural, and pericardial lesions.

sur·gi·cal (ser'ji-kăl). Relating to surgery.

sur·ra (ser'ă). A protozoan disease of camels, horses, mules, dogs, cattle, and other mammals in Africa, Asia, and Central and South America, caused by *Trypanosoma evansi;* infection is generally by mechanical transmission by a bloodsucking species of *Stomoxys* or *Tabanus.* SEE ALSO murrina. [East Indian name]

sur·re·nal (ser-rē'năl). SYN suprarenal (1).

sur·ro·gate (ser'ŏ-gāt). **1.** A person who functions in another's life as a substitute for some third person such as a relative w[illegible] ass[illegible] the nurturing and other responsibilities of the abs[illegible] pare[illegible] **2.** A person who reminds one of another person so t[illegible] one uses the first as an emotional substitute for the second. [L. *surrogo,* to put in another's place]

mother s., one who substitutes for or takes the place of [illegible] mother.

sur·sa·nure (ser-sā'nūr). A superficially healed ulcer, with p[illegible] beneath the surface. [Fr., fr. L. *super,* over, + *sanus,* healthy]

sur·sum·duc·tion (ser-sŭm-dŭk'shŭn). SYN supraduction. [L. *sursum,* upward, + *duco,* pp. *-ductus,* to draw]

sur·sum·ver·sion (ser-sŭm-ver'zhŭn). The act of rotating t[illegible] eyes upward. [L. *sursum,* upward, + *verto,* pp. *versus,* to turn]

---

sur·veil·lance (ser-vā'lans). **1.** The collection, collation, anal[illegible] sis, and dissemination of data; a type of observational study th[illegible] involves continuous monitoring of disease occurrence within [illegible] population. **2.** Ongoing scrutiny, generally using methods distin[illegible] guished by practicability, uniformity, rapidity, rather than con[illegible] plete accuracy. [Fr. *surveiller,* to watch over, fr. L. *super-* + *vigilo,* to watch]

> Surveillance does not aim for accuracy or completeness; rather it is designed to provide practical and uniform results in a timely fashion, so that trends can be spotted and appropriate action taken. Such action might include further investigation of some aspect of an unfolding phenomenon, or even intervention. Surveillance is employed frequently in the monitoring of disease or factors influencing disease. The data being analyzed and interpreted may include 1) mortality and morbidity reports based on death certificates, hospital records, or general practice sentinels or notifications; 2) laboratory test results; 3) disease outbreak reports; 4) vaccine utilization-uptake and side effects; 5) reports of work- or school-related absences due to illness; (6) biological changes in known agents, vectors, or reservoirs of disease.

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immune s., A theory that the immune system destroys tumor cells which are constantly arising during the life of the individual. SYN jmmunological s.

immunological s., SYN immune s.

post-marketing s., procedure implemented after a drug has been licensed for public use, designed to provide information on use and on occurrence of side effects, adverse effects, etc.

sur·vey (ser'vā). **1.** An investigation in which information is systematically collected but in which the experimental method is not used. **2.** a comprehensive examination or group of examinations to screen for one or more findings. **3.** a series of questions administered to a sample of individuals in a population. [O.Fr. *surveeir,* fr. Mediev.L. *supervideo,* fr. *super,* over, + *video,* to see]

field s., the planned collection of data among noninstitutionalized persons in the general population.

skeletal s., radiographic examination of all or selected parts of the skeleton, as for occult fractures, metastases, etc.

sur·vey·ing (ser-vā'ing). In dentistry, the procedure of locating and delineating the contour and position of the abutment teeth and associated structures before designing a removable partial denture.

sur·vey·or (ser-vā'er, ōr). In dentistry, the instrument used in surveying.

sur·viv·al (ser-vī'văl). Continued existence; persistence of life.

sus·cep·ti·bil·i·ty (su-sep-ti-bil'i-tē). **1.** Likelihood of an individual to develop ill effects from an external agent, such as *Mycobacterium tuberculosis,* high altitude, or ambient temperature. **2.** In magnetic resonance imaging, the loss of magnetization signal caused by rapid phase dispersion because of marked local inhomogeneity of the magnetic field, as with the multiple air-soft tissue interfaces in the lung; s. measurement can estimate calcium content in trabecular bone.

sus·pen·sion (sŭs-pen'shŭn). **1.** A temporary interruption of any function. **2.** A hanging from a support, as used in the treatment of spinal curvatures or during the application of a plaster jacket. **3.**

Exhibit A

Fixation of an organ, such as the uterus, to other tissue for support. **4.** The dispersion through a liquid of a solid in finely divided particles of a size large enough to be detected by purely optical means; if the particles are too small to be seen by microscope but still large enough to scatter light (Tyndall phenomenon), they will remain dispersed indefinitely and are then called a colloidal s. SYN coarse dispersion. **5.** A class of pharmacopeial preparations of finely divided, undissolved drugs (*e.g.*, powders for s.) dispersed in liquid vehicles for oral or parenteral use. [L. *suspensio*, fr. *sus-pendo*, pp. *-pensus*, to hang up, suspend]

amorphous insulin zinc s., SYN prompt insulin zinc s.

chromic phosphate P 32 colloidal s., a pure β-emitting colloidal, nonabsorbable radiopharmaceutical administered into body cavities such as the pleural or peritoneal spaces to control malignant effusions. SEE ALSO *sodium* phosphate P 32.

Coffey s., an operative technique following partial excision of the cornu, as in salpingectomy, whereby the broad and the round ligament are sutured over the cornual wound to restore continuity of the peritoneum and to suspend the uterus on the operated side.

crystalline insulin zinc s., SYN extended insulin zinc s.

extended insulin zinc s., a long-acting insulin s., obtained from beef, with an approximate time of onset of 7 hours and a duration of action of 36 hours. SYN crystalline insulin zinc s.

insulin zinc s., a sterile buffered s. with zinc chloride, containing 40 or 80 units per ml; the solid phase of the s. consists of a mixture of 7 parts of crystalline insulin and 3 parts of amorphous insulin. SYN lente insulin.

magnesia and alumina oral s., a mixture of magnesium hydroxide and variable amounts of aluminum oxide; used as an antacid.

prompt insulin zinc s., sterile s. of insulin in buffered water for injection, modified by the addition of zinc chloride such that the solid phase of the s. is amorphous; it contains 40 or 80 units per ml; the duration of action is equivalent to that of insulin injection. SYN amorphous insulin zinc s., semilente insulin.

sus·pen·soid (sŭs-pen'soyd). A colloidal solution in which the disperse particles are solid and lyophobe or hydrophobe, and are therefore sharply demarcated from the fluid in which they are suspended. SYN hydrophobic colloid, lyophobic colloid, suspension colloid. [suspension + G. *eidos*, resemblance]

sus·pen·so·ry (sŭs-pen'sŏ-rē). **1.** Suspending; supporting; denoting a ligament, a muscle, or other structure that keeps an organ or other part in place. **2.** A supporter applied to uplift a dependent part, such as the scrotum or a pendulous breast.

sus·ten·tac·u·lar (sŭs-ten-tak'yū-lăr). Relating to a sustentaculum; supporting.

sus·ten·tac·u·lum, pl. **sus·ten·tac·u·la** (sŭs'ten-tak'yū-lŭm, -lă) [NA]. A structure that serves as a stay or support to another. [L. a prop, fr. *sustento*, to hold upright]

s. li'enis, SYN splenorenal *ligament*.

s. ta'li [NA], support of the talus, a bracket-like lateral projection from the medial surface of the calcaneus, the upper surface of which presents a facet for articulation with the talus.

su·sur·rus (sŭ-ser'ŭs). SYN murmur (1). [L.]

s. au'rium, murmur in the ear.

Sutter blood group. See Blood Groups appendix.

Sutton, Richard L., U.S. dermatologist, 1878–1952. SEE S.'s *disease* (1), *nevus*.

Sutton, Richard L., Jr., U.S. dermatologist, *1908. SEE S.'s *disease* (2), *ulcer*.

SUTURA

su·tu·ra, pl. **su·tu·rae** (sū'tū'ră, -rē) [NA]. SYN suture. [L. a sewing, a suture, fr. *suo*, pp. *sutus*, to sew]

s. corona'lis [NA], SYN coronal *suture*.

sutu'rae cra'nii [NA], SYN cranial *sutures*, under *suture*.

s. ethmoidolacrima'lis [NA], SYN ethmoidolacrimal *suture*.

s. ethmoidomaxilla'ris [NA], SYN ethmoidomaxillary *suture*.

s. fronta'lis [NA], SYN frontal *suture*.

s. frontoethmoida'lis [NA], SYN frontoethmoidal *suture*.

s. frontolacrima'lis [NA], SYN frontolacrimal *suture*.

s. frontomaxilla'ris [NA], SYN frontomaxillary *suture*.

s. frontonasa'lis [NA], SYN frontonasal *suture*.

s. frontozygomat'ica [NA], SYN frontozygomatic *suture*.

s. inci'siva [NA], SYN incisive *suture*.

s. infraorbita'lis, SYN infraorbital *suture*.

s. intermaxilla'ris [NA], SYN intermaxillary *suture*.

s. internasa'lis [NA], SYN internasal *suture*.

s. interparieta'lis, SYN sagittal *suture*.

s. lacrimoconcha'lis [NA], SYN lacrimoconchal *suture*.

s. lacrimomaxilla'ris [NA], SYN lacrimomaxillary *suture*.

s. lambdoi'dea [NA], SYN lambdoid *suture*.

s. meto'pica [NA], SYN metopic *suture*.

s. nasofronta'lis, SYN frontonasal *suture*.

s. nasomaxilla'ris [NA], SYN nasomaxillary *suture*.

s. no'tha (nō'tă), SYN false *suture*. [G. fem. of *nothos*, spurious]

s. occipitomastoi'dea [NA], SYN occipitomastoid *suture*.

s. palati'na media'na [NA], SYN median palatine *suture*.

s. palati'na transver'sa [NA], SYN transverse palatine *suture*.

s. palatoethmoida'lis [NA], SYN palatoethmoidal *suture*.

s. palatomaxilla'ris [NA], SYN palatomaxillary *suture*.

s. parietomastoi'dea [NA], SYN parietomastoid *suture*.

s. pla'na [NA], SYN plane *suture*.

s. sagitta'lis [NA], SYN sagittal *suture*.

s. serra'ta [NA], SYN serrate *suture*.

s. sphenoethmoida'lis [NA], SYN sphenoethmoidal *suture*.

s. sphenofronta'lis [NA], SYN sphenofrontal *suture*.

s. sphenomaxilla'ris [NA], SYN sphenomaxillary *suture*.

s. spheno-orbita'lis, SYN spheno-orbital *suture*.

s. sphenoparieta'lis [NA], SYN sphenoparietal *suture*.

s. sphenosquamo'sa [NA], SYN sphenosquamous *suture*.

s. sphenovomeria'na [NA], SYN sphenovomerine *suture*.

s. sphenozygoma'tica [NA], SYN sphenozygomatic *suture*.

s. squamo'sa [NA], **(1)** SYN squamous *suture*. **(2)** SYN squamoparietal *suture*.

s. squamosomastoi'dea [NA], SYN squamomastoid *suture*.

s. temporozygomat'ica [NA], SYN zygomaticotemporal *suture*.

s. zygomaticofronta'lis, SYN frontozygomatic *suture*.

s. zygomaticomaxilla'ris [NA], SYN zygomaticomaxillary *suture*.

s. zygomaticotempora'lis, SYN zygomaticotemporal *suture*.

su·tur·al (sū'chūr-ăl). Relating to a suture in any sense.

SUTURE

su·ture (sū'chūr). **1.** A form of fibrous joint in which two bones formed in membrane are united by a fibrous membrane continuous with the periosteum. **2.** To unite two surfaces by sewing. SYN stitch (3). **3.** The material (silk thread, wire, catgut, etc.) with which two surfaces are kept in apposition. **4.** The seam so formed, a surgical s. SYN sutura [NA], suture joint. [L. *sutura*, a seam]

absorbable surgical s., a surgical s. material prepared from a substance that can be digested by body tissues and is therefore not permanent; it is available in various diameters and tensile strengths, and can be treated to modify its resistance to absorption and be impregnated with antimicrobial agents.

Albert's s., a modified Czerny s., the first row of stitches passing through the entire thickness of the wall of the gut.

apposition s., a s. of the skin only. SYN coaptation s.

approximation s., a s. that pulls together the deep tissues.

atraumatic s., a s. swaged onto the end of an eyeless needle.

Exhibit A

and his followers that disease was due to an imbalance between solid particles (atoms) of the body and the spaces (pores) between them, a doctrine which opposed the humoral conception of Hippocrates. SYN methodism.

sol·id·ist (sol'i-dist). An adherent of the doctrine of solidism.

sol·id·is·tic (sol-i-dis'tik). Relating to solidism.

sol·i·dus (sol'i-dŭs). That line on a constitution diagram indicating the temperature below which all metal is solid.

sol·i·ped (sol'i-ped). A solid-hoofed animal such as the horse. [L. *solidus*, solid, + *pes*, foot]

sol·ip·sism (sō'lip-sizm, sol'ip-). A philosophical concept that whatever exists is a product of will and the ideas of the perceiving individual. [L. *solus*, alone, + *ipse*, self]

soln. Abbreviation for solution.

sol·u·bil·i·ty (sol-yū-bil'i-tē). The property of being soluble.

sol·u·ble (sol'yū-bl). Capable of being dissolved. [L. *solubilis*, fr. *solvo*, to dissolve]

so·lum (sō'lŭm). Bottom; the lowest part. [L.]

sol·ute (sol'yūt, sō'lūt). The dissolved substance in a solution. [L. *solutus*, dissolved, pp. of *solvo*, to dissolve]

so·lu·tio (sō-lū'shē-ō). SYN solution. [L.]

so·lu·tion (sol., soln.) (sō-lū'shŭn). **1.** The incorporation of a solid, a liquid, or a gas in a liquid or noncrystalline solid resulting in a homogeneous single phase. SEE dispersion, suspension. **2.** Generally, an aqueous s. of a nonvolatile substance. **3.** In the language of the Pharmacopeia, an aqueous s. of a nonvolatile substance is called a solution or liquor; an aqueous s. of a volatile substance is a water (aqua); an alcoholic s. of a nonvolatile substance is a tincture (tinctura); an alcoholic s. of a volatile substance is a spirit (spiritus); a s. in vinegar is a vinegar (acetum); a s. in glycerin is a glycerol (glyceritum); a s. in wine is a wine (vinum); a s. of sugar in water is a syrup (syrupus); a s. of a mucilaginous substance is a mucilage (mucilago); a s. of an alkaloid or metallic oxide in oleic acid is an oleate (oleatum). **4.** The termination of a disease by crisis. **5.** A break, cut, or laceration of the solid tissues. SEE s. of contiguity, s. of continuity. SYN solutio. [L. *solutio*]

acetic s., a vinegar.

amaranth s., a 1% s. of amaranth (trisodium naphthol sulfonic acid), a synthetic vivid red dye, stable in acid and intensified in sodium hydroxide s.; used as a red or pink colorant in liquid pharmaceuticals.

aqueous s., a s. containing water as the solvent; examples include lime water, rose water, saline s., and a large number of s.'s intended for intravenous administration.

Benedict's s., an aqueous solution of sodium citrate, sodium carbonate, and copper sulfate which changes from its normal blue color to orange, red, or yellow in the presence of a reducing sugar such as glucose. SEE ALSO Benedict's *test* for glucose.

Burow's s., a preparation of aluminium subacetate and glacial acetic acid, used for its antiseptic and astringent action on the skin.

chemical s., SEE solution (1).

colloidal s., a dispersoid, emulsoid, or suspensoid. SYN colloidal dispersion.

s. of contiguity, the breaking of contiguity; a dislocation or displacement of two normally contiguous parts.

s. of continuity, division of bones or soft parts that are normally continuous, as by a fracture, a laceration, or an incision. SYN diastasis.

Dakin's s., a bactericidal wound irrigant. SYN Dakin's fluid.

disclosing s., a s. that selectively stains all soft debris, pellicle, and bacterial plaque on teeth; used as an aid in identifying bacterial plaque after rinsing with water.

Earle's s., a tissue culture medium containing $CaCl_2$, $MgSO_4$, $NaHCO_3$, NaCl, $NaH_2PO_4 \cdot H_2O$, and glucose.

ethereal s., a s. of any substance in ether.

Fehling's s., an alkaline copper tartrate s. formerly used for detection of reducing sugars. SYN Fehling's reagent.

ferric ammonium acetate s., a clear, aromatic, reddish-brown liquid which has been used in iron-deficiency anemia in dog and man; a source of iron. SYN Basham's mixture.

Fonio's s., a diluent with magnesium sulfate, used for stained smears of blood platelets.

Gallego's differentiating s., a dilute s. of formaldehyde and acetic acid used in a modified Gram stain to differentiate and enhance the basic fuchsin binding to Gram-negative microorganisms.

Gey's s., a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells.

Hanks' s., a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells; two variations contain $CaCl_2$, $MgSO_4 \cdot 7H_2O$, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $Na_2HPO_4 \cdot 2H_2O$, and D-glucose.

Hartmann's s., SYN lactated Ringer's s.

Hartman's s., a s. used to desensitize dentin in dental operations; contains thymol, ethyl alcohol, and sulfuric ether.

Hayem's s., a blood diluent used prior to counting red blood cells.

Krebs-Ringer s., a modification of Ringer's s., prepared by mixing NaCl, KCl, $CaCl_2$, $MgSO_4$, and phosphate buffer, pH 7.4.

lactated Ringer's s., a s. containing NaCl, sodium lactate, $CaCl_2$(dihydrate), and KCl in distilled water; used for the same purposes as Ringer's s. SYN Hartmann's s.

Lange's s., a colloidal gold s. used to demonstrate protein abnormalities in spinal fluid. SEE Lange's *test*.

Locke-Ringer s., a s. containing NaCl, $CaCl_2$, KCl, $MgCl_2$, $NaHCO_3$, D-glucose, and water; used in the laboratory for physiological and pharmacological experiments.

Locke's s.'s, s.'s containing, in varying amounts, NaCl, $CaCl_2$, KCl, $NaHCO_3$, and D-glucose; used for irrigating mammalian heart and other tissues, in laboratory experiments; also used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells.

Lugol's iodine s., an iodine-potassium iodide s. used as an oxidizing agent, for removal of mercurial fixation artifacts, and also in histochemistry and to stain amebas.

molecular dispersed s., SYN dispersoid.

Monsel s., ferric subsulfate s. used to coagulate superficial bleeding such as that following skin biopsy.

normal s., SEE normal (3).

ophthalmic s.'s, sterile s.'s, free from foreign particles and suitably compounded and dispensed for instillation into the eye.

Ringer's s., **(1)** a s. resembling the blood serum in its salt constituents; it contains 8.6 g of NaCl, 0.3 g of KCl, and 0.33 g of $CaCl_2$ in each 1000 ml of distilled water; used topically for burns and wounds; **(2)** a salt s. usually used in combination with naturally occurring body substances (*e.g.*, blood serum, tissue extracts) and/or more complex chemically defined nutritive s.'s for culturing animal cells. SEE Ringer's *injection*.

saline s., **(1)** a s. of any salt; SYN salt s. **(2)** specifically, an isotonic sodium chloride s.; 0.85 to 0.9/100 ml water.

salt s., SYN saline s. (1).

saturated s. (sat. sol., sat. soln.), a s. that contains all of a substance capable of dissolving; a solution of a substance in equilibrium with an excess undissolved substance.

standard s., standardized s., a s. of known concentration, used as a standard of comparison or analysis.

supersaturated s., a s. containing more of the solid than the liquid would ordinarily dissolve; it is made by heating the solvent when the substance is added, and on cooling the latter is retained without precipitation; addition of a crystal or solid of any kind usually results in precipitation of the excess solute, leaving a saturated s.

test s., a s. of some reagent, in definite strength, used in chemical analysis or testing.

Tyrode's s., a modified Locke's s.; it contains 8 g of NaCl, 0.2 g of KCl, 0.2 g of $CaCl_2$, 0.1 g of $MgCl_2$, 0.05 g of NaH_2PO_4, 1 g

Exhibit A

SO

Genentech Inc., 927 F.2d at 1576, 18 U.S.P.Q.2d 1001, 1010 (Fed. Cir. 1991); *In re Donohue*, 632 F.2d 123, 125-26, 207 U.S.P.Q. 196, 199 (C.C.P.A. 1980); *In re Wiggins*, 488 F.2d 538, 179 U.S.P.Q. 421, 424 (C.C.P.A. 1970). The accompanying Exhibits clearly establish that, at the time of the subject invention, those skilled in the art accept that Factor VIII compositions, either purified or recombinant, contain albumin.

In order to provide insight into the state of the art with respect to blood factor compositions and the use of albumin to stabilize such compositions, the applicant is submitting herewith a number of Exhibits which establish that the standard practice of those skilled in the art is to stabilize Factor VIII compositions with albumin. The original filing date for the subject application was January 19, 1995. The applicant is submitting various Exhibits showing that, at the time of the subject invention, it was believed by those skilled in the art that, for highly labile Factor VIII, albumin was necessary as a stabilizer. The exhibits include the following:

1) Alpha Therapeutic Corporation advertisement in February 15, 2000 issue of the journal *Blood*:

> ...all licensed recombinant Factor VIII products contain albumin, which is necessary for preserving the factor proteins in recombinant products. (emphasis added).

2) Excerpt from 1999 Physicians Desk Reference describing the Helixate® recombinant Factor VIII product:

> The preparation is stabilized with albumin (Human) and *lyophilized.*

3) U.S. Patent No. 4,361,509 describing the stabilization of purified porcine Factor VIII:

> Preparations of VIII: c obtained from a porcine plasma source should be stabilized within 5 to 10% human serum albumin prior to storage.
> Column 10, lines 1-3.

4) Expert Delcaration of Dr. Alan Mackenzie:

> Factor VIII preparations which were derived from human blood necessarily contained albumin. Although there were significant health risks associated with administering albumin to a patient, namely the potential risk for viral contaminants to be present, the presence of albumin was believed to be necessary in order to stabilise the Factor

> VIII protein. Recombinant Factor VIII preparations were also being prepared, but again it was believed to be necessary to add albumin to the preparations to stabilise the proteins. The presence of albumin was believed to be necessary because Factor VIII proteins are extremely labile, even in the presence of other stabilisers.

The applicant is also submitting herewith the following additional Exhibits which further establish that, at the time of the subject invention (and even years later), those skilled in the art believed that albumin was necessary to stabilize Factor VIII preparations:

A. The current University of North Carolina Hemophilia Center Web Site (www.med.unc.edu):

> Monoclonal antibody purified Factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, anti-HBc, ALT, anti-HTLV I/II, and anti-HCV. The Factor VIII is purified by affinity chromatography using mouse monclonal antibodies to human Factor VIII. The purified Factor VIII prior to formulation has a specific activity of ~3000 units per mg. Human albumin is used as a stabilizer in the formulation of the Factor VIII.
>
> . . .
>
> Recombinant Factor VIII. Recombinant Factor VIII is a synthetic form of Factor VIII prepared in mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Because both products are formulated with human albumin, even though the albumin is pasturized, recombinant Factor VIII is subject to the same recalls as plasma-derived Factor VIII in the event that donors with Creutzfeld-Jacob disease contribute to the source plasma from which the albumin is prepared. Two forms of recombinant Factor VIII are currently licensed for use. (emphasis added)

B. Brownlee *et al.* article:

> Both recombinant Factor VIII products (Genetics Institute of Genentech), after purification by immuno-affinity chromatography with monoclonal antibodies, ion-exchange chromatography and other methods, were reported to be essentially indistinguishable from plasma-derived Factor VIII [45, 48]. The full-length recombinant Factor VIII produced by both companies may be regarded as a "first-generation" product, since the CHO cells used to produce Factor VIII were grown in a tissue culture medium that contained protein additives. Moreover, the resultant purified Factor VIII was stabilized by the addition of human albumin. (emphasis added)

C. Puget Sound Blood Center Web page (www.psbc.org; revised November 1999)

Concentrates differ in the purification procedures. Highly purified Factor VIII, e.g. preparations purified over a monoclonal antibody column or current recombinant Factor VIII concentrates, are stabilized by adding 98% of pasteurized human albumin. (emphasis added)

D. Emory Health Sciences Press Release (www.emory.edu; October 10, 2000)

In the early 1990s, scientist carried safety one step further with genetically engineered recombinant Factor VIII products made by inserting the Factor VIII gene into a cell line and producing mass quantities of concentrated human Factor VIII. Although these products contained no human or animal products, they were stabilized with a small amount of albumin, a human blood component. (emphasis added)

E. National Hemophilia Foundation Website (www.hemophilia.org; January 10, 2001)

ReFacto is the first recombinant Factor VIII product formulated without human serum albumin in its final formulation. (emphasis added)

F. U.S. Patent No. 6,171,825 (filed September 4, 1998)

For labile proteins such as Factor VIII, human albumin has been added as a stabilizer during the preparation and purification procedures. Although the albumin is subjected to a viral inactivation step by pasteurization, it would be ideal if recombinant Factor VIII could be manufactured in the complete absence of human and animal blood proteins.

G. WO 94/07510 (published April 14, 1994)

A formulation with a low amount of protein will generally lose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang *et al.*, *J. of Parenteral Sci.* and Tech. Vol. 42, Number 2S, supplement. 1988). Human albumin is also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of Factor VIII is known and is currently used in all highly purified Factor VIII products on the market. (emphasis added)

However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.

EXHIBIT 1

blood

JOURNAL OF
THE AMERICAN
SOCIETY OF
HEMATOLOGY

VOLUME 95
NUMBER 4
FEBRUARY 15, 2000

Natural anticoagulants and sepsis

Sickle cell day hospitals

PCR detection of haptoglobin anaphylaxis

P53 and CML blast crisis

Blood 15/2/00 issue

Questions hemophilia patients are asking:

"Do recombinant Factor VIII products contain human blood elements?"

Yes, they do. The factor proteins themselves are not derived from human blood. They are made from animal cells. However, all licensed recombinant Factor VIII products contain albumin, which is necessary for preserving the factor proteins in recombinant factor products. Albumin is derived from pooled human plasma, much the same way as plasma-derived factor products.

Today there are many effective safeguards against viral contamination of products made from human plasma. Safeguards Alpha Therapeutic Corporation® uses in manufacturing its plasma-derived factor products include affinity chromatography, solvent detergent treatment, heat treatment and nanofiltration. These kinds of safeguards mean that both plasma-derived and recombinant factor products are very safe and effective.

For more information about Alpha Therapeutic Corporation® coagulation factor products, write to: Alpha Therapeutic Corporation, 5555 Valley Boulevard, Los Angeles, CA 90032 or call toll free 1 (800) 292-6118 or visit our web site at www.alphather.com.

alpha®
THERAPEUTIC CORPORATION

EXHIBIT 2

Gammar-P I.V.—Cont.

10) Allow the product vial to remain undisturbed for 5 minutes after diluent addition. Do not touch or mix during this time.

11) After 5 minutes, mix the product vial by gently swirling the vial without creating excessive foam. Never shake the product vial.

Note: A syrup-like layer may remain on the bottom of the vial following reconstitution. Swirl gently to disperse this layer until a homogenous solution is obtained.

12) Examine solution. All unreconstituted product should dissolve with gentle swirling and the solution should be clear and ready to administer in 20 minutes or less.

13) Product contains no preservative. Infusion must be initiated within 3 hours of reconstitution. If not used within this time frame, it should be properly disposed of and not administered.

14) Reconstituted product does not need to be filtered. If a filter is used, it should be a 15 micron filter or larger.

15) If several doses of Immune Globulin Intravenous (Human), Gammar®-P I.V., are to be pooled aseptically for administration, avoid excessive formation of foam in the pooling container and gently swirl the pooling container to mix. **DO NOT SHAKE THE POOLING CONTAINER.**

Administration

CAUTION: When entering the product stopper with an IV set spike for administration, care should be taken to follow the path made by the transfer spike (see Reconstitution).

Immune Globulin Intravenous (Human), Gammar®-P I.V., is to be administered by intravenous infusion. The infusion should begin at a rate of 0.01 mL/Kg/minute, increasing to 0.02 mL/Kg/minute after 15 to 30 minutes. Most patients tolerate a gradual increase to 0.03 – 0.06 mL/Kg/minute. For the average 70 kg person this is equivalent to 2 to 4 mL/minute. If adverse reactions develop, slowing the infusion rate will usually eliminate the reaction. Discard any unused solution.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

HOW SUPPLIED

Individual Vial Packages

Immune Globulin Intravenous (Human), Gammar®-P I.V., is supplied in single dose vials, with diluent and sterile, vented transfer spike for reconstitution. The 10 g dosage form package also contains an administration set. The following dosage forms are available:

(See table at top of previous page)

Bulk Package

Immune Globulin Intravenous (Human), Gammar®-P I.V., 5 g immune globulin/vial is supplied in a bulk pack (NDC 0053-7486-06) of six (6) single dose vials. Each single dose vial should be reconstituted with 100 mL Sterile Water for Injection, U.S.P. (not supplied).

STORAGE

When stored at temperatures not exceeding 25°C (77°F), Immune Globulin Intravenous (Human), Gammar®-P I.V., is stable for the period indicated by the expiration date on its label. Avoid freezing which may damage container for the diluent.

CAUTION: FEDERAL LAW PROHIBITS DISPENSING WITHOUT PRESCRIPTION.

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Revised: June, 1997 IBM 12173

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Antihemophilic Factor (Recombinant)

HELIXATE® ℞

DESCRIPTION

Antihemophilic Factor (Recombinant), HELIXATE® is a sterile, stable, purified, non-pyrogenic, dried concentrate which has been manufactured by recombinant DNA technology. HELIXATE is intended for use in therapy of classical hemophilia (hemophilia A). HELIXATE is produced by Baby Hamster Kidney (BHK) cells into which the human factor VIII (FVIII) gene has been introduced.[1] HELIXATE is a highly purified glycoprotein consisting of multiple peptides including an 80 kD and various extensions of the 90 kD subunit. It has the same biological activity as FVIII derived from human plasma. In addition to the use of the classical purification methods of ion exchange chromatography and size exclusion chromatography, monoclonal antibody immunoaffinity chromatography is utilized along with other steps designed to purify recombinant factor VIII (rAHF) and remove contaminating substances. The final preparation is stabilized with Albumin (Human) and lyophilized. The concentration of HELIXATE is approximately 100 IU/mL. The product contains no preservatives.

Each vial of HELIXATE contains the labeled amount of rAHF in international units (IU). One IU, as defined by the World Health Organization standard for blood coagulation factor VIII, human, is approximately equal to the level of factor VIII activity found in 1.0 mL of fresh pooled human plasma. The final product when reconstituted as directed contains the following excipients: 10–30 mg glycine/mL, not more than (NMT) 500 ug imidazole/1000 IU, NMT 600 ug polysorbate 80/1000 IU, 2–5 mM calcium chloride, 100–130 mEq/L sodium, 100–130 mEq/L chloride, and 4–10 mg Albumin (Human)/mL. HELIXATE must be administered by the intravenous route.

CLINICAL PHARMACOLOGY

The clinical trial of HELIXATE has included 168 patients, enrolled over a 55-month period. A total of 16,186 infusions have been utilized in this trial. The study was conducted in several stages.

Initial pharmacokinetic studies were conducted in 17 asymptomatic hemophilic patients, comparing pharmacokinetics of plasma-derived Antihemophilic Factor (Human) (pdAHF) and HELIXATE.[2] The mean biologic half-life of rAHF was 15.8 hours. The mean biologic half-life of pdAHF in the same individuals was 13.9 hours. A similar degree of shortening of the activated partial thromboplastin time was seen with both rAHF and pdAHF. The mean in vivo recovery of rAHF was similar to pdAHF, with a linear dose-response relationship. The recovery and half-life of rAHF was consistent with initial results following 13 weeks of exclusive treatment with HELIXATE. Subsequently, 826 recovery studies were conducted in 58 hemophilic patients participating in later clinical studies. Mean recovery from this group was 2.48% per IU/kg infused.

Fourteen (14) subjects from initial pharmacokinetic studies commenced home treatment with rAHF. Forty-four (44) additional subjects were then enrolled who treated themselves at home exclusively with rAHF. A total of 12,730 infusions have been administered under this portion of the study, of which 1,021 were given in clinic for recovery studies, 7,239 were given for treatment of bleeds, 4,361 were given as prophylaxis, 5 for minor surgery not requiring hospitalization, and 4 for unspecified reason.

Forty-eight (48) patients have received rAHF on 63 occasions for surgical procedures or in-hospital treatment of serious hemorrhage. Eleven (11) received rAHF for the first time in this study, while 37 were already on study or study participants under an investigation of previously untreated patients. Hemostasis has been satisfactory in all cases, with no adverse reactions.

In a study of previously untreated patients, a total of 3,254 infusions have been administered to 96 patients over a 48-month enrollment period. Hemostasis was successfully achieved in all cases.

During the analytical characterization of Antihemophilic Factor (Recombinant), HELIXATE®, analyses for carbohydrate structure revealed the presence of terminal galactose α1—3 galactose residues. Since naturally occurring antibody to this structure has been reported in humans, a trial in 18 patients was performed in which the half-life and recovery of rAHF with high levels of this carbohydrate residue was compared to that with HELIXATE, which contains no levels of this structure. As in the normal population, all patients had preexisting endogenous antibody to galactose α1—3 galactose in titers ranging from 1:220 to 1:5120 and no significant change in antibody level was noted during the study. While the mean recovery for HELIXATE in the study, 2.76%/IU/kg (N = 43), was significantly different from that of rAHF with high levels of residues, 2.43%/IU/kg (N = 11; p = 0.0001), the recovery for rAHF with high levels of galactose α1—3 galactose is not significantly different from the 2.48%/IU/kg recovery obtained in the larger study from the 58 patients treated with HELIXATE mentioned above. Based on these results, the galactose α1—3 galactose residue appears to have no clinical significance.

INDICATIONS AND USAGE

HELIXATE is indicated for the treatment of classical hemophilia (hemophilia A) in which there is a demonstrated deficiency of activity of the plasma clotting factor, factor VIII. HELIXATE provides a means of temporarily replacing the missing clotting factor in order to correct or prevent bleeding episodes, or in order to perform emergency and elective surgery in hemophiliacs.

HELIXATE can also be used for treatment of hemophilia A in certain patients with inhibitors to factor VIII. In clinical studies of HELIXATE, patients who developed inhibitors to study continued to manifest a clinical response when inhibitor titers were less than 10 Bethesda Units (B.U.) per mL. When an inhibitor is present, the dosage requirement for factor VIII is variable. The dosage can be determined only by clinical response, and by monitoring of circulating factor VIII levels after treatment (see **DOSAGE AND ADMINISTRATION**).

HELIXATE does not contain von Willebrand's factor and therefore is not indicated for the treatment of von Willebrand's disease.

CONTRAINDICATIONS

Due to the fact that Antihemophilic Factor (Recombinant) contains trace amounts of mouse protein (maximum 30 ng/IU rAHF) and hamster protein (maximum 0.04 ng/IU rAHF), HELIXATE should be administered with caution to individuals with previous hypersensitivity to pdAHF or known hypersensitivity to biologic preparations with trace amounts of murine or hamster proteins.

Assays to detect seroconversion to mouse and hamster protein were conducted on all patients on study. No patients developed specific antibody titers against these proteins after commencing study, and no allergic reactions have been associated with rAHF infusions. Although no reactions were observed, patients should be warned of the theoretical possibility of a hypersensitivity reaction, and alerted to the early signs of such a reaction (e.g., hives, generalized urticaria, wheezing and hypotension). Patients should be advised to discontinue use of the product and contact the physician if such symptoms occur.

WARNINGS

None.

PRECAUTIONS

General

HELIXATE is intended for the treatment of bleeding disorders arising from a deficiency in factor VIII. This deficiency should be proven prior to administering HELIXATE.

The development of circulating neutralizing antibodies to factor VIII may occur during the treatment of patients with hemophilia A. In a study of previously untreated patients, inhibitor antibodies have developed in 17 of the 92 patients (18.5%) who have had at least one follow-up titer. The incidence of antibodies is 15/56 (26.7%) in patients with severe disease (<2% factor VIII), 2/18 (11%) in patients with moderate disease (2–5% factor VIII) and 0/18 in patients with mild disease (>5% factor VIII). Ten of the antibodies were high titer (>10 Bethesda Units), three were low titer, and four were low titer and transient. Studies most closely resembling the design of the study of inhibitor development with Antihemophilic Factor (Recombinant), HELIXATE® have reported incidences of inhibitor formation ranging between 18.4 and 52% for patients treated with pdAHF. The incidence of inhibitor formation in previously untreated patients treated with HELIXATE appears to be consistent with that reported in the literature, however the true immunogenicity of HELIXATE is not known at present. Patients treated with rAHF should be carefully monitored for the development of antibodies to rAHF by appropriate clinical observation and laboratory tests.

Product administration and handling of the infusion set and needles must be done with caution. Percutaneous puncture with a needle contaminated with blood can transmit infectious virus including HIV (AIDS) and hepatitis. Obtain immediate medical attention if injury occurs.

...from needles in sharps container after single use. Discard all equipment including any reconstituted HELIXATE product in accordance with biohazard procedures.

Carcinogenesis, Mutagenesis, Impairment of Fertility

In vitro evaluation of the mutagenic potential of HELIXATE failed to demonstrate reverse mutation or chromosomal aberrations at doses substantially greater than the maximum expected clinical dose. In vivo evaluation of rAHF using doses ranging between 10 and 40 times the expected clinical maximum also indicated that HELIXATE does not possess a mutagenic potential. Long-term investigations of carcinogenic potential in animals have not been performed.

Pediatric Use

HELIXATE has been proven to be safe and efficacious in newborns and children while under investigation as previously treated (n=21) and previously untreated patients (n=35) (see CLINICAL PHARMACOLOGY and PRECAUTIONS).

Pregnancy Category C

Animal reproduction studies have not been conducted with HELIXATE. It is also not known whether HELIXATE can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. HELIXATE should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

During the clinical studies conducted in previously treated patients, 47 out of 12,932 infusions (0.36%) were associated with 58 reported minor adverse reactions. Of these, 19 reactions were local to the injection site (e.g., burning, pruritus, erythema); and 39 were systemic complaints (dizziness, nausea, chest discomfort, sore throat, cold feet, unusual taste in mouth, and slight decrease in blood pressure). In the study with previously untreated patients, 3,254 infusions have been associated with 11 minor adverse reactions (0.34%): two reports of erythema at the injection site, one of facial flushing related to the infusion, one report of diarrhea, two reports of nonspecific rash, two reports of fever, and three reports of emesis. No serious reactions have been reported, and all reactions have been self-limited.

DOSAGE AND ADMINISTRATION

Each bottle of HELIXATE has the rAHF content in international units per bottle stated on the label of the bottle. The reconstituted product must be administered intravenously by either direct syringe or drip infusion. The product must be administered within 3 hours after reconstitution.

General Approach to Treatment and Assessment of Treatment Efficacy

The dosages described below are presented as general guidance. It should be emphasized that the dosage of HELIXATE required for hemostasis must be individualized according to the needs of the patient, the severity of the deficiency, the severity of the hemorrhage, the presence of inhibitors, and the factor VIII level desired. It is often critical to follow the course of therapy with factor VIII level assays. The clinical effect of HELIXATE is the most important element in evaluating the effectiveness of treatment. It may be necessary to administer more HELIXATE than would be estimated in order to attain satisfactory clinical results. If the calculated dose fails to attain the expected factor VIII levels, or if bleeding is not controlled after administration of the calculated dosage, the presence of a circulating inhibitor in the patient should be suspected. Its presence should be substantiated and the inhibitor level quantitated by appropriate laboratory tests. When an inhibitor is present, the dosage requirement for rAHF is extremely variable and the dosage can be determined only by the clinical response.

Some patients with low titer inhibitors (<10 B.U.) can be successfully treated with factor VIII without a resultant anamnestic rise in inhibitor titer.[7] Factor VIII levels and clinical response to treatment must be assessed to insure adequate response. Use of alternative treatment products, such as Factor IX Complex concentrates, Antihemophilic Factor (Porcine) or Anti-Inhibitor Coagulant Complex, may be necessary for patients with anamnestic responses to factor VIII treatment and/or high titer inhibitors.

Calculation of Dosage

The in vivo percent elevation in factor VIII level can be estimated by multiplying the dose of rAHF per kilogram of body weight (IU/kg) by 2%. This method of calculation is based on clinical findings by Abildgaard et al.[8] and is illustrated in the following examples:

(See first table above)

(See second table above)

or

(See third table above)

(See fourth table above)

The dosage necessary to achieve hemostasis depends upon the type and severity of the bleeding episode, according to the following general guidelines:

Mild Hemorrhage

Mild superficial or early hemorrhages may respond to a single dose of 10 IU per kg,[9] leading to an in vivo rise of approximately 20% in the factor VIII level. Therapy need not be repeated unless there is evidence of further bleeding.

Moderate Hemorrhage

For more serious bleeding episodes (e.g., definite hemarthrosis, known trauma), the factor VIII level should be raised to 30–50% by administering approximately 15–25 IU per kg. If further therapy is required, a repeat infusion can be given at 12–24 hours.[10]

Severe Hemorrhage

In patients with life-threatening bleeding or possible hemorrhage involving vital structures (e.g., central nervous system, retropharyngeal and retroperitoneal spaces, iliopsoas sheath), the factor VIII level should be raised to 80–100% of normal in order to achieve hemostasis. This may be achieved with an initial rAHF (Antihemophilic Factor (Recombinant), HELIXATE®) dose of 40–50 IU per kg and a maintenance dose of 20–25 IU per kg every 8–12 hours.[11, 12]

Surgery

For major surgical procedures, the factor VIII level should be raised to approximately 100% by giving a preoperative dose of 50 IU/kg. The factor VIII level should be checked to assure that the expected level is achieved before the patient goes to surgery. In order to maintain hemostatic levels, repeat infusions may be necessary every 6 to 12 hours initially, and for a total of 10 to 14 days until healing is complete. The intensity of factor VIII replacement therapy required depends on the type of surgery and postoperative regimen employed. For minor surgical procedures, less intensive treatment schedules may provide adequate hemostasis.[11, 12]

Prophylaxis

Factor VIII concentrates may also be administered on a regular schedule for prophylaxis of bleeding, as reported by Nilsson et al.[13]

Reconstitution

Vacuum Transfer

1. Warm the unopened diluent and the concentrate to room temperature (NMT 37°C, 99°F).
2. After removing the plastic flip-top caps (Fig. A), aseptically cleanse the rubber stoppers of both bottles.
3. Remove the protective cover from the plastic transfer needle cartridge with tamper-proof seal and penetrate the stopper of the diluent bottle (Fig. B).
4. Remove the remaining portion of the plastic cartridge, invert the diluent bottle and penetrate the rubber seal on the concentrate bottle (Fig. C) with the needle at an angle.

   Alternate method of transferring sterile water: With a sterile needle and syringe, withdraw the appropriate volume of diluent and transfer to the bottle of lyophilized concentrate.
5. The vacuum will draw the diluent into the concentrate bottle. Hold the diluent bottle at an angle to the concentrate bottle in order to direct the jet of diluent against the wall of the concentrate bottle (Fig. C). Avoid excessive foaming.
6. After removing the diluent bottle and transfer needle (Fig. D), swirl continuously until completely dissolved (Fig. E).
7. After the concentrate powder is completely dissolved, withdraw solution into the syringe through the filter needle which is supplied in the package (Fig. F). Replace the filter needle with the administration set provided and inject intravenously.
8. If the same patient is to receive more than one bottle, the contents of two bottles may be drawn into the same syringe through a separate unused filter needle before attaching the vein needle.

(See figures A-F in next column)

Rate of Administration

The rate of administration should be adapted to the response of the individual patient, but administration of the entire dose in 5 to 10 minutes or less is well tolerated. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

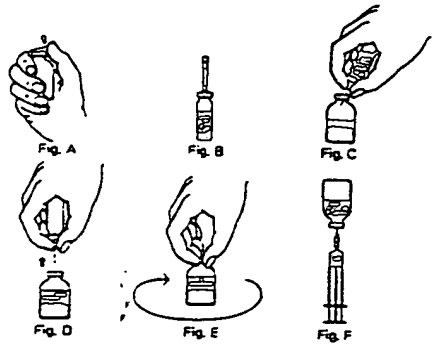
Expected % factor VIII increase = $\frac{\text{\# units administered} \times 2\%/\text{IU/kg}}{\text{body weight (kg)}}$

Example for a 70 kg adult: = $\frac{1400 \text{ IU} \times 2\%/\text{IU/kg}}{70 \text{ kg}}$ = 40%

Dosage required (IU) = $\frac{\text{body weight (kg)} \times \text{desired \% factor VIII increase}}{2\%/\text{IU/kg}}$

Example for a 15 kg child: = $\frac{15 \text{ kg} \times 100\%}{2\%/\text{IU/kg}}$ = 750 IU required

Product Code	Approximate Factor VIII Activity	Diluent
NDC 0053-8120-01	250 IU	2.5 mL
NDC 0053-8120-02	500 IU	5 mL
NDC 0053-8120-04	1000 IU	10 mL



HOW SUPPLIED

Antihemophilic Factor (Recombinant), HELIXATE® is supplied in the following single use bottles with the total units of factor VIII activity stated on the label of each bottle. A suitable volume of Sterile Water for Injection, USP, a sterile double-ended transfer needle, a sterile filter needle, and a sterile administration set are provided.

(See fifth table above)

STORAGE

HELIXATE should be stored under refrigeration (2°–8°C; 36°–46°F). Storage of lyophilized powder at room temperature (up to 25°C or 77°F) for 3 months, such as in home treatment situations, may be done without loss of factor VIII activity. Freezing should be avoided, as breakage of the diluent bottle might occur. Do not use beyond the expiration date indicated on the bottle.

CAUTION

U.S. federal law prohibits dispensing without prescription.

LIMITED WARRANTY

A number of factors beyond our control could reduce the efficacy of this product or even result in an ill effect following its use. These include improper storage and handling of the product after it leaves our hands, diagnosis, dosage, method of administration, and biological differences in individual patients. Because of these factors, it is important that this product be stored properly, and that the directions be followed carefully during use.

No warranty, express or implied, including any warranty of merchantability or fitness is made. Representatives of the Company are not authorized to vary the terms or the contents of the printed labeling, including the package insert for this product, except by printed notice from the Company's headquarters. The prescriber and use of this product must accept the terms hereof.

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Continued on next page

EXHIBIT 3

United States Patent [19]

Zimmerman et al.

[11] 4,361,509

[45] Nov. 30, 1982

[54] ULTRAPURIFICATION OF FACTOR VIII USING MONOCLONAL ANTIBODIES

[75] Inventors: Theodore S. Zimmerman; Carol A. Fulcher, both of La Jolla, Calif.

[73] Assignee: Scripps Clinic and Research Foundation, La Jolla, Calif.

[21] Appl. No.: 330,105

[22] Filed: Dec. 14, 1981

[51] Int. Cl.³ C07G 7/00

[52] U.S. Cl. ..... 260/112 B; 424/101; 424/85

[58] Field of Search ..... 260/112 B; 424/101, 424/85

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Primary Examiner—Howard E. Schain

[57] ABSTRACT

A method of preparing high purity procoagulant protein comprising the steps of (a) adsorbing a VIII:C-/VIII:RP complex from a plasma or commercial concentrate source of factor VIII onto agarose beads bound to a monoclonal antibody specific to VIII:RP, (b) eluting VIII:C with a salt solution, (c) adsorbing the eluted VIII:C on an animohexyl agarose column and eluting the VIII:C with a salt solution.

16 Claims, No Drawings

ULTRAPURIFICATION OF FACTOR VIII USING MONOCLONAL ANTIBODIES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to a method of separating and purifying factor VIII procoagulant activity protein. More specifically, high purity factor VIII procoagulant activity protein is separated from von Willebrand Factor by a two step chromatographic adsorption and concentration technique from plasma or concentrate.

2. Description of the Prior Art

The isolation of the antihemophilic factor from blood plasma has been described in the literature. The precise structure of the antihemophilic factor, also referred to as factor VIII procoagulant activity protein (factor VIII), has not yet been identified, due in part to the unavailability of sufficient quantities of pure material with which to conduct further studies. The limited availability of pure material and its existence in a dilute state has also hindered its use in therapeutic applications.

Factor VIII procoagulant activity protein functions to correct the clotting defect in hemophilic plasma. It circulates in plasma complexed with the von Willebrand factor protein. The latter can alter the platelet function defect in von Willebrand's disease. That portion of the factor VIII von Willebrand factor complex having coagulant activity is referred to as factor VIII procoagulant activity protein, factor VIII-clotting activity or simply VIII:C (the designation of "VIII:C" will be used hereinafter to identify the portion of the factor VIII molecule with such clotting activity.) The other portion of the factor VIII von Willebrand factor complex having the ability to correct the platelet function defect in von Willebrand's disease is referred to as von Willebrand factor, factor VIII-related antigen, VIIIR:Ag, VIII:RP factor. (The description "VIII:RP" will be used hereinafter to identify the platelet correction function of the factor VIII molecule). Although yet unproven, there is evidence to support the conclusion that VIII:C exhibits properties and the behavior of a small molecule which is combined with VIII:RP as a non-covalent complex. There is also a basis for the contention that the properties associated with both VIII:C and VIII:RP may also be a single molecule which under appropriate conditions may be cleaved, yielding two fragments.

In view of the need for identifying the structures of the factor VIII/von Willebrand factor complex, VIII:C and VIII:RP and the important pharmaceutical value of the coagulant activity ascribable to VIII:C, numerous attempts have been made to purify factor VIII and to separate and concentrate VIII:C and VIII:RP. The techniques used are based generally on either immunoadsorption or ion exchange chromatography. Such techniques as heretofore used have had limited success due to the difficulty of desorbing the proteins from the charged ionic material in an undamaged condition or recovering same in suitable quantities.

One such method for separating VIII:C from VIII:RP utilizing immunoadsorbent chromatography has been reported by E. G. D. Tuddenham et al, "The Properties of Factor VIII Coagulant Activity Prepared by Immunoadsorbent Chromatography", JOURNAL OF LABORATORY CLINICAL MEDICINE, Vol. 93, p. 40 (1979). The reported method is a one-step separation of VIII:C from nearly all VIII:RP and from most other plasma proteins employing a chromatographic column packed with agarose beads to which polyclonal antisera to VIII:RP (anti-VIII:RP) are coupled. Factor VIII/von Willebrand factor containing plasma is passed through the column which adsorbs both VIII:C and VIII:RP. Other unwanted plasma proteins are removed from the column by washing with buffered saline solution and the desired VIII:C is obtained by subsequent elution with a calcium-ion gradient. Although it is stated to be an improvement in both purity and yield of VIII:C, when compared to the previously known methods, it is also stated that the resulting product also contains VIII:RP and other plasma proteins. Such contaminants may be attributable to the use of polyclonal antisera bound to the agarose beads. Since a majority of the immunoglobulins from which the antisera are constituted are not specific to VIII:RP, the effective number of sites where antibodies specific to VIII:RP may be bound to agarose is relatively small due to competition between the antisera for a finite number of bonding sites on the agarose.

Another method for separating VIII:C from VIII:RP and ristocetin co-factor by a chromatographic technique employing aminohexyl-substituted agarose has been described by D. E. G. Austen, "The Chromatographic Separation of Factor VIII on Aminohexyl Sepharose," BRITISH JOURNAL OF HAEMATOLOGY, Vol. 43, p. 669 (1979). The described method is stated to be an improved method for the component parts of both human and porcine factor VIII/von Willebrand factor. This method, however, also suffers from the fact that contaminants are present in the resulting product. In both the Tuddenham et al and Austen methods a contaminated product, which is more dilute than is normally desired, is formed.

Hence, it is clear that there still exists a need for an improved method for separating and purifying VIII:C from VIII:RP using plasma or concentrates. Therefore, it is an object of the present invention to satisfy such a need.

SUMMARY OF THE INVENTION

The present invention relates to a method of separation of the component molecules of the factor VIII/von Willebrand factor complex, VIII:C and VIII:RP, and the purification and concentration of the pro-coagulant activity protein VIII:C. The method achieves the object of producing highly purified VIII:C using a two step procedure.

The first step involves immunoadsorption of factor VIII from plasma or a commercial concentrate. The adsorbent employed comprises a monoclonal antibody specific to VIII:RP which is bound to a suitable substrate such as, agarose beads. After the VIII:C/VIII:RP is initially adsorbed, the substrate particles are washed extensively with a buffer solution to remove unadsorbed protein. The adsorbed material is then treated with a calcium ion containing solution to elute the adsorbed VIII:C. The VIII:RP portion remains adsorbed on the anti-VIII:RP bound material. At this point about 40–60% of the VIII:C initially adsorbed is recovered in a highly purified state. However, the procoagulant activity protein recovered, although extremely pure, i.e., largely free from contaminants, is too dilute to be of significant therapeutic value.

The second step of the present process is directed to substantially concentrating the recovered purified VIII:C using a technique which may be characterized as affinity chromatography.

The VIII:C solution obtained from the first step of the present process having a potency of approximately 10–20 International Units (hereinafter "units") is processed in a column containing aminohexyl substituted agarose. The column is then washed with a buffer solution and the VIII:C is eluted with a calcium ion-containing solution to yield a VIII:C concentration in excess of 1000 units per ml, and being greater than 160,000 fold purified from plasma. Thus, the present method yields unexpectedly high purity procoagulant activity protein in a highly concentrated and therapeutically useful state. Methods used heretofore fail to achieve such notable results for several reasons. The method of Tuddenham et al, described earlier, employs bound polyclonal antisera instead of the specific and highly selective monoclonal antibodies to VIII:RP as used in the present invention. As a result, fewer specific antibodies to VIII:RP are coupled for a given weight of agarose. In the method of the present invention monoclonal antibodies are exclusively bound to a relatively inert substrate. When the method of Tuddenham et al is used only 2.6 to 6.4 units of VIII:RP per ml of immunoglobulin-agarose beads (equivalent to 53.1–82.9% of the amount applied to the column) are removed. This compares to greater than 1000 units per ml of beads (or 90–100% of the VIII:RP which is applied to the column) which is recovered when the monoclonal antibody immunoadsorbent of the present invention is employed. This ability to adsorb more VIII:C/VIII:RP (factor VIII/von Willebrand factor) per ml of beads accordingly results in a higher concentration of VIII:C when it is subsequently eluted from the immunoadsorbent. Thus, 10–20 units of VIII:C per ml of eluant are obtained with the present invention, in contract to 0.5–1.25 units per ml of eluant with the Tuddenham et al method.

The present method also permits the selection of a monoclonal antibody having a high affinity for VIII:RP; however, the use of polyclonal antibodies results in varying affinities. It should be realized that there is an indirect relationship between the affinity of the bound antibody for VIII:RP and the elution of VIII:RP. Thus, the higher the affinity of the antibody for VIII:RP, the less VIII:RP will be present with VIII:C in the eluant. The present invention also makes it possible to produce an unlimited supply of the specified monoclonal antibody, thus eliminating variations among different batches.

Although Austen, as earlier described, has reported the use of aminohexyl-agarose to separate VIII:C from VIII:RP, such a material has not heretofore been used to concentrate VIII:C following a separation and purification step. Heretofore, the highest VIII:C concentrations achieved by using aminohexyl agarose in chromatography were 0.53 units per ml of eluant for human protein and 2.38 per ml of eluant for porcine VIII:C. The present method permits concentrations several orders of magnitude greater than these. Perhaps of even greater significance, is the fact that the present invention provides for a greater purification of human VIII:C than has ever been reported (164,000 vs 17,000 fold over plasma). The present method, which is described in more detail hereinafter, yields VIII:C with a specific activity of 2,360 units/mg when commercial concentrate is used. This corresponds to a 164,000 fold purification from plasma. The ratio of VIII:C to VIII:RP is greater than 10^5 as compared to the ratio in plasma.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The following description provides details of the manner in which the embodiments of the present invention may be made and used in order to achieve the separation, purification and concentration of VIII:C to a degree of purity and concentration not known heretofore. This description, while exemplary of the present invention, is not to be construed as specifically limiting the invention and such variations which would be within the purview of one skilled in this art are to be considered to fall within the scope of this invention.

A. Preparation of Monoclonal Antibody to VIII:RP

The monoclonal antibody to VIII:RP which is subsequently bound to the separation substrate may be prepared in a stepwise procedure starting with a highly purified preparation of factor VIII/von Willebrand factor (VIII:C/VIII:RP complex). The purification for immunization is accomplished with material obtained from a plasma source. Less highly purified material for coating polyvinyl plates is obtained in higher concentration from commercial extracts such as FACTORATE (trademark of Armour Pharmaceutical Co., Tuckahoe, N.Y.) or Hemophil (trademark of Hyland Laboratories, Costa Mesa, California). Purification is performed by a standard agarose-gel filtration of cryoprecipitate, such as that described by Zimmerman and Roberts, "Factor VIII Related Antigen", appearing in IMMUNOASSAYS: CLINICAL LABORATORY TECHNIQUES FOR THE 1980's, R. M. Nakamura et al, eds., Alan R. Liss, Inc., New York, pp. 339–349 (1980). Mice were injected with highly purified factor VIII/von Willebrand factor obtained from plasma according to the following procedure. On day zero, the mice are injected intraperitoneally with a composition prepared by dissolving (or suspending) 10 Mg of the protein in 0.1 ml of buffer containing 0.05 M Tris, 0.15 M sodium chloride, 0.02% sodium azide, 1 mM phenyl methyl sulfonyl fluoride, trasylol 10 units/ml at pH7.3, and shaking with an equal volume of complete Freund's adjuvant. On day 14, the mice are again injected with the same material except that incomplete Freund's adjuvant is substituted for complete Freund's adjuvant. On day 21, the injection of day 14 is repeated. On day 38, the mice are injected with purified VIII:C/VIII:RP only. On day 42, the spleens of the mice are removed and fused according to a standard procedure, of the type described by J. P. Brown et al "Protein Antigens of Normal and Malignant Human Cells Identified by Immunoprecipitation with Monoclonal Antibodies", JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 225, pp. 4980–4983 (1980). The standard technique is varied only to the extent that 35% polyethylene glycol 1000 is substituted for 50% polyethylene glycol. A radioimmunoassay method for clones producing antibody to VIII:RP is performed according to the following procedure. Polyvinyl plates with a "V" bottom, flexible type are coated with 0.1 ml of factor VIII purified from commercial extract according to the procedure indicated above and having a concentration of 0.125 mg/ml of protein. The plates are blocked with albumin, washed with buffer and incubated with the culture fluids from the clones to be tested. The plates

are then washed and reacted with rabbit anti-mouse IgG antiserum, washed a second time and ^{125}I labeled goat anti-rabbit IgG antiserum is added to the wells and incubated. The plates are again washed, then dried and the wells cut-out and counted. After determining the clones which are positive they are subcloned at least twice and stable clones producing antibody to VIII:RP are then injected into the peritoneal cavities of Balb/C mice which have been pretreated intraperitoneally with 0.5 ml of pristane at least four days prior to injection of cells. Hybridoma cells are injected at concentrations of approximately 5×10^6 cells per mouse in 0.5 ml of Delbecco's modified Eagle's medium without fetal bovine serum. The mice are tapped when bloated and ascites fluid is collected in heparin at approximately 10 units/ml. Ascites fluid from multiple mice is pooled to provide a convenient volume for subsequent isolation of the monoclonal IgG. If the heparinized ascites fluid is not used immediately, it may be stored at −70° C. and thawed just prior to use. The final yield of IgG from the ascites fluid is approximately 1 g of IgG per 100 ml of ascites fluid.

The specificity of the monoclonal IgG for the purpose of purifying VIII:C may be assessed by coupling the IgG to a separation substrate medium, in the manner described hereinafter, and demonstrating that the bound IgG removes both VIII:RP and VIII:C from plasma and that the VIII:C may be subsequently eluted with a solution containing calcium ions while the VIII:RP remains complexed to the monoclonal IgG which is bound to the solid-state substrate.

The monoclonal IgG, which is to be used subsequently to prepare the immunoadsorbent, may be isolated from heparinized pooled ascites fluid immediately after collection or a frozen portion of the stored solution may be thawed. Regardless of whether fresh or frozen material is used, the solution is brought to 4° C. and treated with an equal volume of phosphate buffered saline solution (PBS), the composition of which is set forth below. The diluted ascites is precipitated by dropwise addition with stirring at 4° C. of an equal volume of saturated ammonium sulfate (SAS); prepared by boiling an excess of ammonium sulfate in water, cooling to 4° C., filtering undissolved crystals and adjusting the pH to 7.0 with ammonium hydroxide. The precipitate and its supernatant liquid are stirred for at least 2 hours and centrifuged at 4° C. Centrifugations are preferably carried out at 14,000 rpm for 60 minutes (30,000×g). The supernatant solution of ascites is precipitated twice more with SAS and the mixture of precipitate and supernatant liquid stirred and centrifuged in the same manner as in the first cycle. The pellets resulting from the third precipitation are resuspended in a volume of PBS equal to that of the diluted ascites fluid and then disalyzed exhaustively against PBS. Clots appearing in the dialysis bags are removed by centrifugation at 20° C. The dialyzed IgG is adsorbed by stirring it with a 5% aqueous solution of aluminum hydroxide at room temperature and centrifuging at 20° C. after adsorption. The adsorption treatment is repeated at least three more times using 2.5% aluminum hydroxide solution for each treatment after the first. The adsorbed IgG is brought to 4° C. and reprecipitated once with SAS as described above. The precipitated pellets may be stored at −20° C. until used.

B. Preparation of the Immunoadsorbent

The immunoadsorbent is prepared by suitably preparing the monoclonal IgG for coupling, preparing the solid substrate for coupling and reacting the two components to bind the former to the later.

(i) Preparation of IgG for Coupling

Either freshly precipitated IgG may be used or previously frozen precipitate may be thawed for use. The material is then dialyzed against PBS, and while still in the PBS, the volume and IgG concentration ($A_{280}/1.4$=mg./ml IgG) are determined. The IgG is then treated with between 10 and 30 microliters, preferably 20 microliters, of diisopropylfluorophosphate per 50 ml of IgG solution. The resulting solution is stirred at room temperature in a hood for 30 minutes and the treated IgG, immediately prior to use, is dialyzed overnight against coupling buffer. The coupling buffer found most suitable is a 0.25 M sodium bicarbonate solution adjusted to a pH of 9, preferably with sodium hydroxide.

(ii) Preparation of Solid Substrate for Coupling

Although the monoclonal antibody may be bound to any material which does not have a high affinity for protein, particularly factor VIII itself, such materials as glass beads, agarose and derivatives thereof are preferred. Most preferred is a crosslinked agarose available commercially as a gel known as Sepharose CL2B (trademark of Pharmacia Fine Chemicals, Piscataway, N.J.).

The method of preparing the preferred immunoadsorbent resin is generally the same as that disclosed in the literature, such as the method of J. Porath et al, JOURNAL OF CHROMATOGRAPHY, Vol. 86, pp. 53–56 (1973). The method found most suitable is as follows: a volume of about 2 liters of Sepharose CL2B is placed in an acid-cleaned 2 liter sintered glass filter funnel. The resin is washed with water and filtered to a moist cake. The washed resin is placed in a large (approximately 4 liter) glass beaker equipped with a magnetic stirring bar. To the resin is then added 750 ml of cold potassium phosphate buffer solution, prepared by mixing one part of a 5 M dibasic potassium phosphate solution with two parts of 5 M tribasic potassium phosphate solution. Sufficient cold water is added to bring the final volume to 3 liters. The mixture is then chilled to 4° C. and maintained at between 4°–10° C. in an ice-water bath placed on a magnetic stirring plate. In a hood, cyanogen bromide is added to 300 ml of water in a stoppered glass bottle containing a magnetic stirring bar. The mixture is rapidly stirred until solution results. The cyanogen bromide solution is then added with stirring over a 2 minute period to the cold Sepharose mixture. Stirring is continued for an additional 8 minutes and then transferred to a chilled 2 liter sintered glass filter funnel supported in a 4 liter vacuum flask. The cyanogen bromide treated resin is then washed with approximately 20 liters of cold water or until the pH of the filtrate is neutral. The washed resin is then quickly equilibrated with cold coupling buffer and then transferred to a 4 liter plastic beaker equipped with a large magnetic stirring bar.

(iii) Coupling the Monoclonal Antibody to the Solid Substrate

The solid substrate resin, prepared as indicated above, is ready to be used when it is equibrated with coupling buffer and should not be stored thereafter. Accordingly, the resin mixture is combined with the

IgG which was previously dialyzed overnight against coupling buffer. The combined resin/IgG suspended mixture is stirred at 4° C. for a period of about 24 hours. The A_{280} of an undiluted sample of the supernatant coupling liquid may be determined using bovine serum albumin (BSA) as a standard or Bio-Rad protein assay (Bradford reagent) with BSA as standard. The percentage ligand which is coupled may then be calculated. When the above described procedure is followed, this is usually about 95%. Any remaining active sites on the resin not coupled to antibody may be blocked by washing the resin on a sintered glass filter funnel with cold coupling buffer containing 0.1 M glycine. The resin is then resuspended in this solution to a final volume equal to that when the resin and antibody, each in coupling buffer, were combined. The suspension is stirred slowly overnight at 4° C. The resin is then washed thoroughly with VIII:C-buffer, the composition of which is given below. The coupled, blocked resin is then pre-eluted with VIII:C-buffer additionally containing 0.5 M calcium ions, preferably calcium chloride. The resin is again washed with VIII:C buffer alone and stored at 4° C. or in a continuously pumped column at room temperature until ready for use. The coupling density of IgG to SEPHAROSE should be 2–5 g, preferably 3–4 g IgG/liter of SEPHAROSE.

C. Separation and Purification of VIII:C

(i) Sample preparation of factor VIII, such as human and animal plasmas and commercial concentrates of factor VIII, may be employed in the present invention and the method is not limited as to a particular type of material. Preferred materials, and those which have demonstrated successful results, are porcine and human plasmas and commercially available concentrates of human factor VIII, such as FACTORATE available from Armour Pharmaceutical Co. The following description provides details for using both porcine plasma or commercial human concentrate such as FACTORATE:

FACTORATE is reconstituted by adding 25 ml portions of VIII:C-buffer to the contents of each of 20 bottles containing 400–500 VIIIC units per bottle (25 ml per bottle). The mixture is adjusted to a final volume of 1 liter with VIII:C-buffer. A sample aliquot of 0.5 ml may be removed for assay and the remaining material applied to the immunoadsorbent column overnight at a rate of approximately 60 ml/hour.

Porcine plasma, when not freshly drawn, is citrated by conventional means and stored frozen. When ready to be used it is thawed at a temperature of between 35°–40° C., preferably 37° C. and applied directly to the column at 60 ml/hour.

It should be noted that while the description of the present invention refers, and is directed primarily, to the use of immunoadsorbent coupled particles in a chromatography column, it is within the scope of this invention to perform batchwise separations by placing the antibody-bound resin particles in a suitable container and after adding reconstituted concentrate or plasma, VIII:C as outlined above and described in more detail below.

When the process is carried out in a chromatography process, the following embodiments are preferred:

The resin is placed in a column, such as an Amicon 86001, (trademark of Amicon Corp., Lexington, Mass.), equipped with a peristaltic pump and a high flow head. When concentrate is used as the source of factor VIII, for 20 bottles of diluted concentrate, approximately 1.5 liters of resin, prepared as indicated above, is used. When porcine plasma is used, 150 ml of resin is used for each liter of plasma.

After the sample is applied to the column, it is washed with 1 liter of VIII:C-buffer, followed by a second washing with VIII:C-buffer which additionally contains 0.5 M NaCl. Approximately 20 liters of saline-buffer is used when factor VIII is applied as concentrate and 20 bed volumes when porcine plasma is employed. Optimum results are obtained with a flow rate of 1 liter/hour.

Elution of purified VIII:C is accomplished with VIII:C-buffer containing calcium ions. Although a linear gradient, as taught by Tuddenham et al, supra, works well, it is not required in order to accomplish the object of this invention; a solution having a fixed calcium ion concentration is quite adequate. Thus, when VIII:C derived from concentrate is being eluted, VIII:C-buffer, 0.25 to 0.5 M with respect to calcium chloride, preferably 0.35 M, is used advantageously as a flow rate of between 450 to 750 ml/hour and preferably 600 ml/hour. When the VIII:C is obtained from porcine plasma, elution is performed with VIII:C-buffer being a calcium chloride concentration of between 0.35 and 0.7 M, preferably 0.5 M and at a flow rate of between 10 and 30 ml/hour, preferably 20 ml/hour. Fractions of 12 ml and 3 ml are collected for VIII:C originating from concentrate and porcine plasma, respectively. Those fractions containing at least 1.0 unit/ml of VIII:C activity are pooled and the total volume and activity of the pool determined.

The VIII:C pool is initially concentrated to 10–20 ml by a standard procedure such as pressure ultrafiltration. For this purpose, Amicon stirred cell in which a YM-10 membrane under 50 psi of nitrogen pressure has been found to work well. Slow stirring is continued for 30 minutes after nitrogen pressure is released, and the volume and activity of the concentrated pool are determined. The pool may be stored for a brief period, that is, overnight for example, if a temperature of 4° C. is maintained.

It may be noted that the immunoadsorbent column described above may be regenerated by treatment of the column with 2 bed volumes of 3 M aqueous sodium thiocyanate solution run at a flow rate of about 0.5–1 liter/hour to elute VIII:RP.

D. Concentration of Purified VIII:C

Although the VIII:C recovered from the separation from VIII:RP by means of the immunoadsorbent column is highly purified, it is still too dilute to be therapeutically useful. Further concentration and a four fold increase in purification when porcine plasma is used is accomplished by use of an aminohexyl agarose column which is prepared and used in the following manner:

(i) Preparation and/or Conditioning of a Aminohexyl Agarose Column:

Aminohexyl agarose is agarose which has been reacted with 1,6-diaminohexane to yield an agarose resin having a number of 6 carbon atom chains, each of which has a terminal amino group. It may be prepared according to the method described by Austen, supra, or acquired from a commercial supplier. One such material which has been used successfully in the present invention is available under the name of AH-SEPHAROSE 4B (trademark of Pharmacia Fine Chemicals, Piscataway, N.J.).

Whether prepared or purchased, the resin should be conditioned prior to use. This may be accomplished as follows, the volumes, amounts and dimensions being adjusted in proportion to the amount of material to be concentrated:

Approximately 1 gram of aminohexyl agarose (AH-SEPHAROSE 4B) is placed in a sintered glass filter funnel and washed with at least 200 ml of 0.5 M sodium chloride, while stirring. The resin is then equilibrated with VIII:C-buffer and packed in a column of approximately 0.9 cm diameter. A Bio-Rad Econo-Column with flow adapters has proven quite suitable for the type of use considered here. The bed volume of the packed column is approximately 4 ml.

(ii) Application to and Use of the Aminohexyl Agarose Column

The concentrated pool, prepared as described above, is diluted 1:10 in VIII:C-buffer to a final concentration of 100–200 ml when using the amounts of resin and column size as described in the immediately preceding section. The diluted pool is applied to the column at a flow rate of 200 ml/hour.

The column is then washed with VIII:C-buffer which contains calcium ions, preferably from calcium chloride. The solution should be between 0.01 M to 0.03 M, preferably 0.025 M with respect to calcium ions.

Elution of the concentrated VIII:C is achieved at a flow rate of between 5 to 20 ml/hour, preferably 10 ml/hour with VIII:C-buffer containing a higher concentration of calcium ions than was employed with the preceding washing step. Again, calcium chloride is the preferred source of calcium ions in a concentration of between 0.25 to 0.5 M, preferably 0.3 M. Fractions of 1 ml volume are collected and assayed as described below. Collected fractions may be stored at 4° C. or frozen. Preparations of VIII:C obtained from a porcine plasma source should be stabilized within 5 to 10% human serum albumin prior to storage.

Assays may be performed by diluting the fractions with VIII-C buffer if necessary and further diluting the fraction 1:100 in assay buffer prior to addition to the substrate. A standard partial thromboplastin time assay is employed.

The composition of the buffer solutions is as follows:

Phosphate Buffered Saline Solution:

1.6 g sodium phosphate, monobasic monohydrate
8.4 g sodium phosphate, dibasic anhydrous
61.4 sodium chloride
Water to 7 liters
pH of buffer is 7.2

VIII:C-Buffer

ml 0.02 M imidazole
ml 0.15 M sodium chloride
ml 0.10 M lysine
ml 0.02% sodium azide

pH of buffer is adjusted with concentrated hydrochloric acid to 6.8.

The data listed hereinafter in Tables I and II are representative of that obtained according to the present invention, as described above.

TABLE I

VIII:C Obtained From FACTORATE Concentrate as the Source of VIII:C/VIII:RP

	Volume (ml)	VIIIC (Units/ml)*	VIIIC (Total Units)	Protein (mg/ml)	Protein (Total mg)	Recovery (%)	Specific Activity (Units/mg)	From Plasma (Fold Purif.)
Sample Applied to Immunoadsorbent	500	18.8	9400	29	14,500	—	0.7	50
Pool resulting from Immunoadsorbent	1020	4.6	4692	—	—	50	—	—
Pool After Initial Concentration	20	134	2680	—	—	29 (57)	—	—
Sum Resulting from Aminohexyl Column	—	—	1576	—	—	17 (59)	—	—
Aminohexyl Fraction #3	0.95	1172	1112	0.51	0.48	12	2294	163,857
Aminohexyl Fraction #4	—	545	—	0.23	—	—	2370	169,285

*A frozen human plasma pool used as the standard for VIIIC assays and assigned the value of 1 human unit per ml.

TABLE II

VIII:C Obtained From Citrated Porcine Plasma

	Volume (ml)	VIIIC (Units/ml)	VIIIC (Total Units)	Protein (mg/ml)	Protein (Total mg)	Recovery (%)	Specific Activity (Units/mg)	From Plasma (Fold Purif.)
Sample Applied to Immunoadsorbent	1000	1*	1000	76	76,000	100	0.013	—
Pool Resulting from Immunoadsorbent	70	8.8	613	—	—	61	—	—
Pool After Initial Concentration	5.76	88	494.5	0.242	1.355	49.5	364	28,000
Sum Resulting from Aminohexyl Column	5.0	49	247	0.035	0.175	25	1413	109,000

*Porcine plasma used as the standard for VIIIC assays and assigned the value of 1 porcine VIIIC unit per ml.

Although only preferred embodiments are specifically illustrated and described herein, it will be appreciated that many modifications and variations of the present invention are possible in light of the above teachings and within the purview of the appended claims without departing from the spirit and intended scope of the invention.

What is claimed is:

1. An improved method of preparing Factor VIII procoagulant activity protein comprising the steps of

(a) adsorbing a VIII:C/VIII:RP complex from a plasma or commercial concentrate source onto particles bound to a monoclonal antibody specific to VIII:RP,

(b) eluting the VIII:C,

(c) adsorbing the VIII:C obtained in step (b) in another adsorption to concentrate and further purify same,

(d) eluting the adsorbed VIII:C, and

(e) recovering highly purified and concentrated VIII:C.

2. A method according to claim 1, wherein the elutant used in each of steps (b) and (d) is a saline solution.

3. The method according to claim 2, wherein the saline solution is calcium chloride.

4. The method according to claim 3, wherein the concentration of said calcium chloride solution used in steps (b) and (d) ranges from about 0.25 M to about 0.5 M.

5. The method according to claim 1, wherein said adsorbent particles in step (a) are agarose.

6. The method according to claim 1, wherein aminohexyl agarose is employed in step (c) as the adsorbent.

7. The method according to claim 6, wherein calcium chloride solution is employed as the elutant in steps (b) and (d), concentration of said calcium chloride solution ranging from about 0.25 M to about 0.5 M in step (b) and from about 0.25 M to about 0.5 M in step (d).

8. An improved immunoadsorbent for isolation and purification of VIII:C from VIII:C/VIII:RP comprising a monoclonal antibody specific to VIII:RP bound to solid particles.

9. The improved immunoadsorbent of claim 8, wherein said solid particles comprise a resin.

10. The improved immunoadsorbent of claim 9, wherein said resin comprises agarose.

11. The improved immunoadsorbent of claim 10, wherein said agarose is cross-linked agarose.

12. The improved immunoadsorbent of claim 11, wherein said immunoadsorbent has a coupling density of 3 to 4 g of monoclonal antibody per liter of agarose.

13. Highly purified and concentrated VIII:C prepared in accordance with the method of claim 1.

14. Highly purified and concentrated VIII:C prepared in accordance with the method of claim 6.

15. In a method for purifying Factor VIII procoagulant activity protein from plasma or concentrate, the improvement comprising the step of passing said plasma or concentrate through a chromatographic type column having adsorbent to which is bound monoclonal antibodies which is specific to VIII:RP and eluting the VIII-C therefrom.

16. The method according to claim 15, wherein said adsorbent is agarose and said elutant is a saline solution.

* * * * *

EXHIBIT 4

Home > AlphaNine®SD/Alphanate®/Profilnine®SD

ANTIHEMOPHILIC FACTOR (HUMAN)
ALPHANATE®
Solvent Detergent/Heat Treated

DESCRIPTION

Antihemophilic Factor (Human), Alphanate®, Solvent Detergent/Heat Treated, is a single dose, sterile, lyophilized concentrate of Factor VIII (AHF) intended for intravenous administration in the treatment of hemophilia A, or acquired Factor VIII deficiency.

Alphanate® is prepared from pooled human plasma by cryoprecipitation of the Factor VIII, fractional solubilization, and further purification employing heparin-coupled, cross-linked agarose which has an affinity to the heparin binding domain of vWf/FVIII:C complex.[1] The product is treated with a mixture of tri(n-butyl) phosphate (TNBP) and polysorbate 80 to reduce the risks of transmission of viral infection. In order to provide an additional safeguard against potential non-lipid enveloped viral contaminants, the product is also subjected to a 60°C heat treatment step for 72 hours. However, no procedure has been shown to be totally effective in removing viral infectivity from coagulation factor products.

Alphanate® is labeled with the antihemophilic factor potency (Factor VIII:C activity) expressed in International Units (IU) per vial, which is referenced to the WHO International Standard.

Alphanate® contains Albumin (Human) as a stabilizer, resulting in a final container concentrate with a specific activity of at least 5 IU FVIII:C/mg total protein. Prior to the addition of the Albumin (Human) stabilizer, the specific activity is significantly higher.

When reconstituted with the appropriate volume of Sterile Water for Injection, USP, Alphanate® contains 0.3 - 0.9 g Albumin (Human)/100 mL; NMT 5 mmol calcium/L; NMT 750 µg glycine/IU FVIII:C; NMT 1.0 U heparin/mL; 10 - 40 mmol histidine/L; NMT 0.1 mg imidazole/mL; 50 - 200 mmol arginine/L; NMT 1.0 µg polyethylene glycol and polysorbate 80/IU FVIII:C; NMT 10 mEq sodium/vial; and NMT 0.1 µg TNBP/IU FVIII:C.

CLINICAL PHARMACOLOGY

Antihemophilic Factor (Human) is a constituent of normal plasma and is required for clotting. The administration of Alphanate® temporarily increases the plasma level of this clotting factor, thus minimizing the hazard of hemorrhage.[2,3] Following the administration of Alphanate® during clinical trials, the mean *in vivo* half-life of Factor VIII observed in 12 adult subjects with severe hemophilia A was 17.9 ± 9.6 hours. In this same study, the *in vivo* recovery was 96.7 ± 14.5% at 10 minutes postinfusion.[4] Recovery at 10 minutes postinfusion was also determined as 2.4 ± 0.4 IU FVIII rise/dL plasma per IU FVIII infused/kg body weight.[4]

The solvent detergent treatment process has been shown by Horowitz, et al., to provide a high level of virus kill without compromising protein structure and function.[5] The susceptibility of human pathogenic viruses such as the human immunodeficiency viruses, hepatitis viruses, as well as marker viruses such as sindbis virus and vesicular stomatitis virus (VSV), to inactivation by organic solvent detergent treatment has been discussed in the literature.[6]

Like Kwate BVI of Bayer

EXHIBIT A

Products currently available for use in hemophilia A

Cryoprecipitate. Prepared from single plasma units by cold precipitation and resuspension in plasma. Although the source plasma is screened for HIV 1 and 2, anti-HBc, ALT, and anti-HCV, there is no viral inactivation step for the cryoprecipitate normally obtained from your local blood bank. Because of the theoretical risk of HIV infection, the use of cryoprecipitate to treat hemophilia is not recommended. The New York Blood Center is currently working on techniques to treat cryoprecipitate using solvent-detergent techniques but this is not currently available.

Intermediate purity factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, ALT, anti-HBc, anti-HTLV I/II, and anti-HCV. Viral inactivation steps are used in all intermediate purity concentrates although the techniques differ from product to product. These inactivation steps generally appear to be highly effective against HIV and selected hepatitis viruses. The U. S. Hemophilia/HIV Seroconversion Surveillance Project sponsored jointly by the Centers for Disease Control, the Food and Drug Administration, and the National Hemophilia Foundation has shown no HIV seroconversions in hemophilia attributable to factor concentrates since 1987 and no hepatitis B or C seroconversions in hemophilia attributable to factor concentrates since 1992.

Intermediate purity factor VIII products which are currently available:

- **Factor VIII SD** (NY Blood Center; Melville) - Viral inactivation by extraction with TNBP - sodium cholate.
- **Humate-P** (Behringwerke) - Pasturized product prepared by heat treatment in solution to 60°C for 10 hours.
- **Profilate OSD** (Alpha) - Viral inactivation using TNBP - polysorbate 80.
- **Koate HP** (Bayer-Miles) - Viral inactivation using TNBP - polysorbate 80.
- **MelATE** (Melville) - Viral inactivation using TNBP - polysorbate 80.
- **Alphanate** (Alpha) - Purified from plasma by affinity chromatography using heparin-agarose to bind von Willebrand factor which is complexed with factor VIII. Viral inactivation using TNBP - polysorbate 80.

Monoclonal antibody purified factor VIII. Prepared from pooled human plasma which is screened for anti-HIV 1 and 2, anti-HBc, ALT, anti-HTLV I/II, and anti-HCV. The factor VIII is purified by affinity chromatography using mouse monoclonal antibodies to human factor VIII. The purified factor VIII prior to formulation has a specific activity of ~3000 units per mg. Human albumin is used as a stabilizer in the formulation of the factor VIII.

Monoclonal factor VIII products which are currently available:

- **Monoclate-P** (Centeon) - Prepared from commercial source plasma.
- **Hemofil-M** (Baxter) - Prepared from commercial source plasma. Affinity purified using a mouse monoclonal antibody which recognizes the factor VIII heavy chain.
- **AHF-ARC** (Red Cross) - Prepared from Red Cross volunteer source plasma and purified and formulated at Baxter using the same methods used to purify Hemofil-M.

Recombinant factor VIII. Recombinant factor VIII is a synthetic form of factor VIII prepared in mammalian cells, such as Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. These are immortalized cell lines which are stable in culture, and permit the high level expression of human proteins with expected post-translational modifications such as tyrosine sulfation and carbohydrate attachment. Initial concerns about the possible immunogenicity of recombinant factor VIII have been somewhat alleviated by 1) studies in previously-treated patients showing a very low prevalence of inhibitors, 2) studies in previously-untreated patients (PUPs) showing a higher but stable prevalence of inhibitors, and 3) studies in newborn and transgenic mice showing a lack of immunogenicity of recombinant factor VIII compared with plasma factor VIII. Because both products are formulated with human albumin, even though the albumin is pasturized, recombinant factor VIII is subject to the same recalls as plasma-derived factor VIII in the event that donors with Creutzfeld-Jacob disease contribute to the source plasma from which the albumin is prepared. Two forms of recombinant factor VIII are currently licensed for use.

Recombinant factor VIII products which are currently available:

- **Recombinate** (Baxter) - The full length cDNA for factor VIII is used to synthesize Recombinate. Synthesis is in Chinese hamster ovary (CHO) cells and the factor VIII is purified by monoclonal affinity affinity chromatography using a mouse anti-factor VIII antibody which recognizes the 90 kDa heavy chain of factor VIII. The purified factor VIII is formulated with pasturized human albumin as a stabilizer. No viral inactivation step is used in Recombinate. Licensed by the FDA in December 1992.

- **Kogenate** (Bayer-Miles) - The full length cDNA for factor VIII is synthesized in baby hamster kidney (BHK) cells, purified by monoclonal affinity chromatography using a mouse anti-factor VIII antibody that recognizes the x domain of factor VIII, and formulated with pasturized human albumin. No viral inactivation step is used in Kogenate. Licensed by the FDA in March 1993.

- **Bioclate** (Centeon) - identical with Baxter Recombinate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

- **Helixate** (Centeon) - identical with Bayer-Miles Kogenate and distributed by Centeon under a licensing agreement on the patent for factor VIII.

The Medical and Scientific Advisory Committee (MASAC) recommends that individuals with hemophilia and their providers should consider the use of recombinant derived clotting factor as the first choice for replacement therapy.

Click here for more information on product safety and product recalls from the Food and Drug Administration.

Click here to return to the UNC Hemophilia Center Home Page.

EXHIBIT B

Recombinant Protein Drugs
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Clotting factors VIII and IX

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Introduction

Haemophilia is the most common congenital disorder of coagulation and affects approximately 1 in 10,000 males around the world. Haemophilia A is due to a deficiency of factor VIII in the circulating blood whilst haemophilia B (also known as Christmas disease) is a clinically identical disorder caused by factor IX deficiency. It is less common than haemophilia A and affects 1 in about 30,000 males. Both factors VIII and IX are essential glycoproteins in the clotting cascade [1] (Fig. 1). The hallmark of severe haemophilia is recurrent and spontaneous haemarthrosis, typically affecting the hinge joints such as the ankle, knee and elbow. The severity of bleeding depends upon the level of factor in the blood.

Severe haemophilia is usually defined by a level of <2 iu/dl (or <2%) of factor VIII or IX in plasma. Moderately severely affected patients have levels varying from 2–5 iu/dl and mild from 5–25 iu/dl. It is unusual for an infant to have spontaneous haemarthroses in the first few months of life, and the first joint to be affected tends to be the ankle as the child learns to crawl. Repeated bleeding into joints may cause permanent damage, with painful arthritis and limb deformity and associated muscle wasting. Bleeding into muscles is also a feature of haemophilia, but this is usually a consequence of direct injury, albeit often minor. Bleeds into certain areas are particularly dangerous because of the risk of compression of neighbouring structures. Bleeds in the tongue can obstruct the airway, and retroperitoneal bleeding within the ilio-psoas muscle may result in femoral nerve compression. Bleeding from the gastrointestinal tract and bleeding into the urinary tract may also occur. There is also a significant risk of intracranial haemorrhage in severe haemophilia which was a significant cause of mortality in the past when treatment was not so readily available. The poor prognosis in the absence of effective treatment is reflected by the fact that the median life expectancy of haemophiliacs in Sweden was only 11 years during the period 1831–1920, but rose to 56.8 years during the period 1961–1980 [2]. It is also interesting to note that a century ago haemophilia affected several members of the royal families of Britain, Spain and Russia,

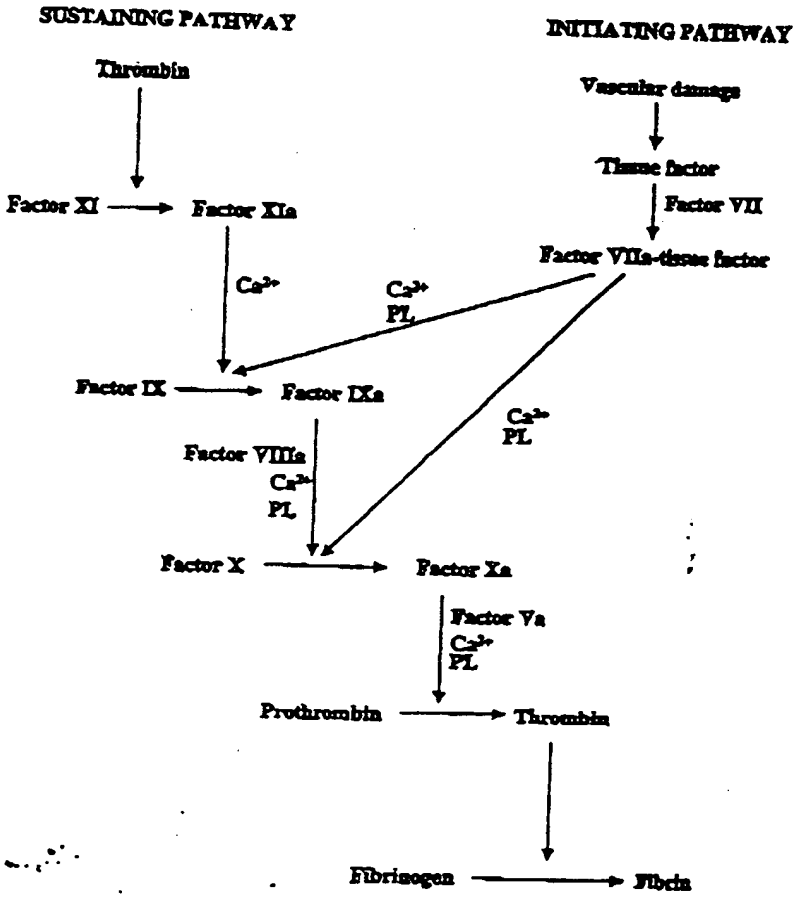

Figure 1. The revised clotting cascade. Thrombin, formed by the action of factor Xa as a result of vascular damage to the initiating (or extrinsic) pathway activates factor XI, thereby sustaining the cascade through the sustaining (or intrinsic) pathway. PL = phospholipid. The scheme is simplified and omits many details for clarity [1].

but, despite the best medical facilities of the age being available, all affected members died in their youth and no surviving descendants are affected.

Approximately 5% of patients with haemophilia A develop inhibitory antibodies to factor VIII at some stage but it is quite likely that this figure underestimates the true prevalence. Inhibitor development in haemophilia B is, by contrast, very rare (<1%). This is a potentially serious complication of therapy, as patients are refractory to conventional doses of coagulation factor concentrates and bleeding can be difficult to control. Family studies suggest that

there is a genetic predisposition to the formation of antibodies but no HLA association or other linkages have been conclusively identified. There is some evidence that people of Afro-Caribbean origin are more susceptible to inhibitor formation than other ethnic groups. Certain mutations are undoubtedly associated with a significantly increased risk of inhibitor development, particularly large gene deletions and nonsense mutations resulting in stop codons – presumably because patients lack immune tolerance [3, 4]. There is a weaker association between inhibitor formation and the presence of an inversion in intron 22 of the factor VIII gene [5].

The genes for factor VIII and IX are both located at the telomere of the long arm of the X chromosome and thus haemophilia is inherited as an X-linked, recessive condition affecting males. The daughters of affected males are obligate carriers but sons are normal. A proportion of all cases of haemophilia occurs in the absence of a previous family history and is due to new mutations. Perhaps the most famous example is that of Queen Victoria, who had a haemophiliac son (Leopold) and also two daughters who were carriers. It is not known, however, whether they suffered from haemophilia A or B. There are very rare instances of haemophilia affecting females due to inheritance of the defective gene from both parents or to unequal X-inactivation in carriers [6]. There are also reports of haemophilia in females with Turner's syndrome (XO karyotype) and congenital androgen insensitivity (testicular feminization with XY karyotype).

Molecular basis of haemophilia A

Factor VIIIa is an essential cofactor which is required for the activation of factor X by factor IXa in the clotting cascade (Fig. 1). Factor VIII is a glycoprotein of 2332 amino acids and is synthesized predominantly by hepatocytes of the liver. It is processed intracellularly in the Golgi apparatus by proteolytic cleavage giving rise to a N-terminal heavy chain and a C-terminal light chain. Its domain structure (Fig. 2A) includes a carbohydrate-rich B domain that is not required for activity (see below). Factor VIII is activated to give rise to factor VIIIa by further proteolytic cleavage, probably by thrombin. In plasma, factor VIII circulates as a large glycoprotein complex non-covalently bound to multimers of von Willebrand factor (Fig. 2). The factor VIII gene is about 186 kb in length, with 26 exons, and is situated on the long arm of the X chromosome at Xq28.

Developments in molecular biology have permitted rapid identification of mutations in haemophilia A (and B) patients by various methods. PCR amplification of either genomic DNA or cDNA derived from the reverse transcription of mRNA of patient lymphocytes has superseded older methods based on restriction enzyme digestion and Southern blotting. Although automated DNA sequence analysers have been developed, gene sequencing of the entire factor VIII gene would be both expensive and labour-intensive because of its size.

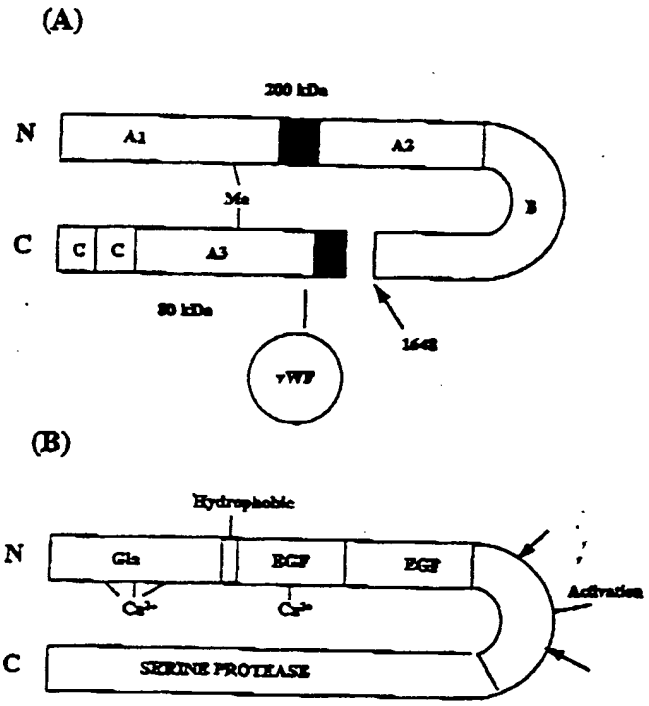

Figure 2. The domain structure of (A) factor VIII and (B) factor IX. In factor VIII (above) the N-terminal heavy chain and C-terminal light chain, formed after proteolysis at amino acid 1648 as shown, are stabilized by a metal (Me) binding site; a proposed site of binding to von Willebrand (vWF) factor is shown. The A1 and 2, B and C domains are marked. In factor IX (below) arrows mark the sites of proteolytic cleavage in the activation domain by either factor XIa or VIIa. For further details, see text.

Thus methods have been developed to initially prescreen the gene or mRNA, in order to define the mutated exon of the DNA, or region of the mRNA, before sequencing to define the actual mutation itself. Chemical cleavage of mismatches in heteroduplexes formed from the mutated DNA and a test DNA is the best current technique for detecting all mutations [7, 8]. Other useful methods are conformation-sensitive gel electrophoresis and denaturing-gradient gel electrophoresis, although these alternatives do not allow all mutations to be detected.

By far the commonest single genetic defect causing haemophilia A is an inversion in intron 22, which is encountered in about 20% of all patients, and in nearly half of all severely affected patients [5]. The inversion mechanism proposed involves homologous, intra-chromosomal recombination between an intronless gene of unknown function, designated F8A, which lies within intron

22 of the factor VIII gene and either of 2 further copies of F8A flanking the factor VIII gene. In either case, the inversion results in a truncated factor VIII gene generating a truncated mRNA and inactive factor VIII protein, thus explaining the severe haemophilia. Recombination with the distal copy of F8A is commoner than with the proximal copy and accounts for approximately 80% of the inversions (reviewed in [9]). It is now recognised that inversion is much more common in a male than a female meiosis.

All other known haemophilia A mutations are summarized in a valuable mutation database [10]. At present, 454 unique mutations are listed, including 92 cases of large deletions. However, it is generally thought that large deletions account for only a minority of cases of haemophilia and the reason that a much higher proportion than 5% is listed in the database reflects an ascertainment bias. Most mutations are point mutants or very short additions or deletions of only a few nucleotides. Nonsense, frameshift and splice-site mutations, caused by point mutations or short additions or deletions, usually cause severe haemophilia. Most other cases are caused by missense mutations causing amino acid substitutions and 218 missense mutations are presently known [10]. They have varying degrees of severity for patients from very mild to very severe [10]. Mutations at CG doublets, giving rise to a TG or CA doublet, are common. As is well known, CG doublets are genomic "hotspots" because of specific methylation of cytosine to 5' methylcytosine and because the methylated cytosine is particularly susceptible to mutation by deamination.

Molecular basis of haemophilia B

Factor IXa is a serine protease required for the activation of factor X in the clotting cascade. Factor IXa is itself formed from factor IX by proteolysis by either factor VIIa-tissue factor complex, which initiates the clotting cascade, or by factor XIa, which sustains it [1] (Fig. 1). Factor IX is a glycoprotein of 415 amino acids and is made up of a N-terminal γ-carboxyglutamic acid-rich sequence (Gla domain), two epidermal growth factor-like (EGF) domains – the first of which binds calcium – an activation domain and finally a C-terminal serine protease or catalytic domain (Fig. 2B). The 12 glutamic acid residues of the Gla domain undergo γ-carboxylation by a vitamin K-dependent γ-glutamyl carboxylase in the endoplasmic reticulum (ER) during the synthesis of factor IX in the liver hepatocyte. This essential post-translational modification is necessary for the correct protein folding and calcium binding of factor IX. The factor IX gene is about 34 kb in length and contains eight exons. Its basic exon structure is similar in organisation to coagulation factors VII, X and protein C and it is likely that they all evolved from a common ancestral gene by gene duplication.

The factor IX gene is considerably smaller than that of factor VIII, and patients with haemophilia B have been studied more extensively than those with haemophilia A. The first defects identified in haemophilia B were large

gene deletions detected in patients with inhibitory antibodies by Southern blotting [3]. However, it is now clear that point mutations, or short additions or deletions, account for the vast majority of cases of haemophilia B, and over 689 unique mutations are listed in an extensive mutation database of 1918 families who have been studied from around the world [6, 11]. Most of the unique mutations are point mutants causing missense, nonsense or splicing defects involving all 8 exons. For example, there are at present 425 different missense mutations that cause amino acid substitutions. These include the original case of Christmas disease, which is a G → C mutation at nucleotide 30,070 causing an amino acid change from Cys → Ser at residue 206 within exon 7 [12]. Many mutations have been observed more than once, even where there is no known kinship. Many of these repeat mutations – as in the case of haemophilia A patients, occur at CG doublets, suggesting they are independent mutation hotspots. However, other repeat mutations do not involve CG doublets, suggesting a founder effect. A good example is the mutation at nucleotide 31,311 where there are 41 examples.

A few patients have been described in whom the factor IX level rises significantly after puberty (the haemophilia B Leiden patients); this is associated with complete clinical recovery. These interesting regulatory mutations have been all localized to a short region of the factor IX promoter and 18 unique mutations are now known [6]. These mutations inhibit the binding of transcription factors, e.g., hepatic nuclear factor 4, which are required for the efficient initiation of transcription. An androgen response element (ARE) in the factor IX promoter, which interacts with the androgen receptor in the presence of testosterone, is responsible for the upregulation of factor IX mRNA at puberty [13]. Consistent with this hypothesis, patients with mutations in the ARE do not recover at puberty [13].

Treatment of haemophilia

Over a century ago, the first effective product available for treatment of bleeding episodes was fresh blood. The preparation of an antihaemophilic factor of bovine origin by Macfarlane in 1954 [14] was a major therapeutic advance, although serious allergic reactions were not infrequent. The use of cold-insoluble cryoprecipitate was introduced in 1965. The subsequent development of lyophilized concentrates of factors VIII and IX in the early 1970s transformed the life of haemophiliacs. The goals of treatment, as stated by the World Federation of Haemophilia, are "to minimise disability and prolong life, to facilitate general social and physical well-being and to help each patient achieve full potential whilst causing no harm" (www.wfh.org). Advances in therapy have certainly resulted in a dramatic increase in the longevity of haemophiliacs in developed countries. A study of 717 Dutch patients documented a calculated life expectancy for these patients of 66 years, compared with 74 year for normal males [15], and the authors of this study concluded

that the mortality associated with even severe haemophilia was similar to that associated with cigarette smoking.

Treatment of bleeding episodes involves the intravenous injection of coagulation factor concentrates. The total dose and frequency of treatment is determined by the severity and site of bleeding. Most bleeds resolve with a single infusion, if the bleed is recognised early and treated promptly. There is an increasing move to prophylactic therapy, in which the patient gives himself injections at home of coagulation factors two or three times a week to prevent bleeds rather than just treating on demand when bleeds occur. Patients on prophylactic therapy experience few or even no spontaneous bleeds and thus progressive joint damage and arthritis can be avoided.

The pooling of plasma donations introduced the risk of transmission of viral infections, and many haemophiliacs were infected with HIV and/or hepatitis C in the 1980s. In the UK, 1229 haemophiliacs were infected with HIV, and about 300 were exposed to hepatitis C [16, 17]. The introduction in 1985 of physical methods of viral inactivation, such as heat-treatment or the addition of a solvent/detergent mixture, eliminated the risk of transmission of HIV or hepatitis C. However, other viruses are relatively resistant to these measures and cases of hepatitis A and parvovirus infection have been documented even with these more modern products. More recently, there has been concern about the possibility of transmission of prion disorders, although no cases have yet been described in haemophilia [18]. Recombinant products offer the greatest margin of safety for haemophiliac patients and have been recommended as the treatment of choice for all patients with haemophilia (see below).

Development of recombinant DNA methods

During the late 1970s there was a quiet revolution in molecular biology. Using restriction enzymes and DNA ligase, it became possible to clone copies of mRNA into either bacterial plasmids or into bacteriophages such as phage λ. Such clones could then be sequenced using Sanger's dideoxy chain termination method or Maxam & Gilbert's chemical degradation procedure. Once these mRNAs had been cloned, each cloned cDNA served as a probe in order to isolate from the entire genome its respective gene, which could then in turn be sequenced. Oligonucleotides were starting to be used as primers for the enzymatic synthesis of DNA. The presence of introns in most genes was discovered. Southern had described his blotting technique that was to become so widely used in this field until the development of PCR techniques much later in the 1980s.

Globin, immunoglobulin and ovalbumin mRNA – all mRNA species which could be purified in reasonable quantities from specialized cells or organs – could now be cloned in *E. coli* and sequenced in their entirety for the first time. Moreover, the new cloning methods suggested that mRNA and/or genes of medical interest, such as human insulin and human growth hormone mRNA,

might be cloned and the protein expressed artificially in bacteria. This, it was thought, might be a cost-effective and safer alternative to isolating these proteins from natural sources for the treatment of diabetes and pituitary dwarfism.

1980 was a time of optimism and some of us thought that we might be able to clone rare, low-abundance mRNA, if only new methods could be developed. We decided to clone human factor IX mRNA. This would be an ambitious and difficult project because of its low abundance in liver. However, if the project were successful, the benefits for patients with haemophilia B would be considerable.

Recombinant factor IX

The factor IX gene was cloned in 1982 – some 2 years before the factor VIII gene – and this work will be described first. The expression of biologically active recombinant factor VIII, however, proved technically easier than factor IX. Moreover the priority was to produce recombinant factor VIII before recombinant factor IX because of the higher incidence of haemophilia A than B in the population. Consequently recombinant factor VIII was available for the treatment of patients in 1992, much sooner than recombinant factor IX, which was only produced much later in 1997.

Cloning the human factor IX gene in Oxford

It was the availability of the complete amino acid sequence of bovine factor IX [19] that initially led us to think that it might be possible to clone the factor IX gene. We thought that short synthetic oligonucleotides, with their sequence partly predicted from the genetic code and the amino acid sequence of the protein, would probably be the key to cloning rare mRNAs, like the factor IX mRNA. We had previously used oligonucleotides both for our earlier sequencing of regions within the α and β-globin, ovalbumin and immunoglobulin mRNAs [20], as well as for our later influenza cloning [21, 22]. Oligonucleotides were clearly powerful and highly specific reagents for nucleic acids. In particular, they could be used to hybridize to specific sequences in mRNA and prime the synthesis of cDNA by reverse transcriptase.

We first decided to enrich the bovine factor IX mRNA, which we had isolated from a calf liver, by two successive sucrose density-gradient centrifugation steps [23]. We then set up a rabbit reticulocyte *in vitro* translation assay to detect factor IX mRNA, locating it in the 20–22S fraction of the sucrose gradient. We estimated that it had been purified about 10-fold over the starting mRNA. Even so it was unlikely that the factor IX mRNA was >0.1% pure at this stage.

We then synthesized, as a mixture, eight 14-nucleotide (nt) long oligonucleotides (oligo N1 mixture), complementary to the bovine mRNA sequence, whose sequence was predicted from the amino acid sequence of residues 348–352 of bovine factor IX (Fig. 3). This was a particularly favourable region

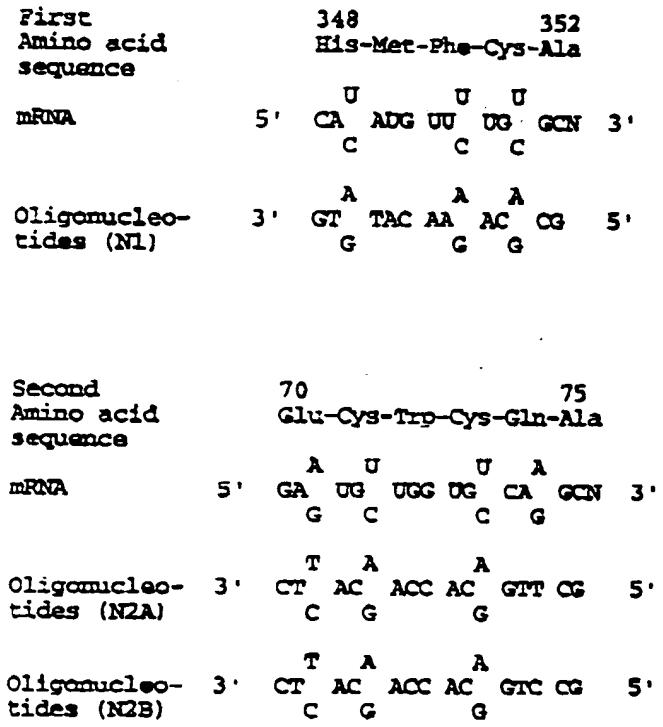

Figure 3. Two regions of amino acid sequences of bovine factor IX used to design mixed oligonucleotide sequence primers (N1) or probes (N2A & N2B) [22]. See text for further details.

of amino-acid sequence since the number of alternative possible nucleotide sequences predicted from the genetic code was limited to 8, because the sequence contains a methionine residue which has a unique codon. This oligo N1 mixture of primers was used to prime the synthesis of cDNA from factor IX-enriched bovine mRNA in order to generate a library of cDNA clones. Briefly, after synthesis of cDNA by reverse transcriptase and subsequent removal of mRNA by alkaline hydrolysis, "loop-back" synthesis of double-stranded DNA was catalysed by *E. coli* DNA polymerase I (Klenow subfragment) in the absence of added primer. Cloning of this double-stranded DNA was simplified by restriction digestion of DNA with MboI (GATC-recognition sequence) and ligation into the unique Bam HI site of the classic plasmid, pBR322. An "MboI" cDNA library of about 7000 recombinant clones was thus obtained in *E. coli*.

Bacterial colonies were then screened on Whatman 541 paper [24] using a second mixture (oligo N2A plus oligo N2B) of 16 ^{32}P-labelled 17-nt long

oligonucleotides based on the predicted sequence of bovine factor IX mRNA of another favourable region of amino acid sequence between residues 70–75 (Fig. 3). We obtained a single clone, labelled BIX-1, which was sequenced by the Maxam and Gilbert method. This clone encoded bovine factor IX mRNA from amino acids 52 to 139 and predicted an amino acid sequence that was in complete agreement with the literature, except for a single discrepancy changing the amino acid at position 57. This discrepancy was, in fact, confirmed as a correction to the published bovine factor IX sequence, since it was also present in an independent clone in a "dC/dG-tailed" cDNA library of about 10,000 *E. coli* colonies.

Thus the choice of oligonucleotides and our ability to synthesize them in-house were two important factors in our initial success in isolating factor IX cDNA clones. Acting in the hope that the human and bovine factor IX genes would be sufficiently conserved in nucleotide sequence to cross-hybridize, the bovine factor IX probe was then used to probe a Charon 4A bacteriophage λ library of human genomic clones [22]. A positively hybridizing clone, λHIX1b, was isolated and mapped by restriction enzyme cleavage and Southern blotting. A restriction fragment corresponding to the probe was thus identified on Southern blots and sequenced. There was 85% nucleotide sequence conservation between bovine and human factor IX gene in the region initially sequenced. We had thus isolated a clone containing part of the human factor IX gene.

Further cloning and expression of factor IX in mammalian cells

Further cloning Two additional reports from other groups [25, 26] extended our initial work by describing the complete coding sequence of human factor IX, which was found to be preceded by a leader sequence with a potential signal peptide and propeptide sequence. There was uncertainty, however, as to whether the entire coding sequence had been cloned, since the 5' non-coding of the factor IX mRNA was not characterized. There were, also, in one report [26] some 6 differences in the coding nucleotide sequence attributed, incorrectly as it turned out later except for one nucleotide [27], to the presence of polymorphisms. Interestingly, although the details of the cloning procedures differed in these reports from our own work, all of the studies had relied on oligonucleotides to prepare or isolate clones from cDNA libraries.

We concentrated on characterizing the complete factor IX mRNA sequence by cDNA cloning, and independently sequencing all exons in order to define splice points and to check for potential cDNA cloning errors, which are known to occur during reverse transcription and cloning. We defined the mRNA start site precisely and sequenced the entire 3' non-coding region of the mRNA [27]. The factor IX gene turned out to be about 34 kb long with 8 exons. The factor IX mRNA was about 2800 nucleotides long and had a relatively short 5' non-coding region, but a reasonably long 3' non-coding sequence of nearly 1400 nucleotides containing the usual AAUAAA poly(A) signal. Finally, the complete human factor IX gene sequence of 33.5 kb, including all 7 introns, was established by Davie's group [28].

Expression of biologically active factor IX in mammalian cells If cloning the factor IX cDNA and gene sequence were to be a "useful" contribution to medicine, then expression of recombinant factor IX protein would have to be achieved. A major uncertainty was whether *biologically active* factor IX was synthesized, because after synthesis, factor IX has to be post-translationally modified and correctly processed. In particular, after γ-carboxylation of 12 glutamyl residues near the N-terminus of factor IX by a vitamin K-dependent carboxylase, the 18 amino acid residues of the propeptide sequence [29] must be cleaved by a furin protease. Both these processes are essential for factor IX activity. It was conceivable that these essential post-translational events would only occur in liver, the site of factor IX synthesis in the body, and would not occur in hepatic or other cell types derived from other tissues in tissue culture.

We were fortunate that the aim of producing biologically active factor IX in mammalian cells in tissue culture was feasible but it was only achieved after a considerable effort by Don Anson and Ian Jones in the Brownlee laboratory. We published, in 1985, that "we were able to isolate small amounts of biologically active (recombinant) human factor IX" [30]. In particular, factor IX was expressed in rat hepatoma cells that had been stably transfected with a factor IX expression plasmid. Much higher yields of >90% biologically active factor IX were obtained in canine kidney cells (MDCK cells) using a slightly different expression plasmid [31]. Our results were particularly convincing because we purified the recombinant factor IX by immuno-affinity chromatography.

Subsequently, higher yields of recombinant factor IX were achieved by other groups using different expression vectors in different mammalian cells, i.e., baby hamster kidney (BHK), human hepatoma (HepG2) and chinese hamster ovary (CHO) cells [32–34]. But in every case the biological activity of factor IX was less than in our reports. Indeed, in the initial work by Kaufman's group in CHO cells (in which the factor IX gene was amplified in the genome) factor IX was secreted to give as much as 100 mg/ml of factor IX in the medium, yet only 2% was biologically active [34]. This low biological activity in CHO cells was subsequently solved by the introduction of a furin-type protease (see below), suggesting that factor IX propeptide processing must have been limiting.

Development of recombinant factor IX for clinical use

The observation that some, albeit low, biological activity was present in the factor IX expressed in CHO cells was important [34], since this cell line has properties that are particularly suited for large-scale culture needed for the industrial production of recombinant proteins by the biotechnology industry. Therefore, development work was undertaken by Genetics Institute, Boston. This presented a significant challenge since it was essential that propeptide processing and γ-carboxylation, known to be essential for factor IX activity, had to occur. Neither of these processes had been previously required in CHO cells in the production of recombinant proteins for the biotechnology industry.

Initially it was suspected that γ-carboxylation was limiting in the factor IX-expressing CHO cells, thus accounting for the presence of the inactive, incompletely γ-carboxylated factor IX. However, when the cloned γ-carboxylase became available, transient transfection of expression constructs of the γ-carboxylase into factor IX-secreting CHO cells failed to improve factor IX biological activity [35]. By contrast, propeptide processing of factor IX was significantly improved by the co-expression of a furin-like enzyme, specifically a soluble form of PACE (paired basic amino acid cleaving enzyme) introduced stably into the CHO cell line [36]. Finally, Genetics Institute produced factor IX in a form that was fully processed by screening factor IX-expressing CHO clones to identify those with the highest factor IX-processing capacity, by optimizing the concentration of vitamin K added to the defined serum-free culture medium (which lacked any added protein) and by defining the precise conditions of growth of the factor IX-secreting CHO clone in large-scale (2500 l) bioreactors.

A down-stream large-scale purification protocol was developed that did not require the use of monoclonal antibodies for affinity purification, to avoid any risk of introducing viral contaminants. The purified recombinant factor IX showed a slightly reduced γ-carboxylation level of about 11 γ-carboxyglutamyl (Gla) residues per molecule compared to 12 Gla residues in blood-derived factor IX (Tab. 1 and ref. [37]). A new result obtained from the analysis of this recombinant factor IX is that the post-translational modification of Gla residues 36 and 40 are not apparently needed for activity [38]. Other differences between recombinant and human blood-derived factor IX were appar-

Table 1. Comparison of post-translational modifications of plasma-derived and recombinant factor IX

Modification	Plasma-derived	Recombinant
1. γ-carboxyglutamic acid (Gla)*		
12 of 12 Gla residues	100%	60%
11 of 12 Gla residues	0%	35%
10 of 12 Gla residues	0%	5%
2. β-hydroxyaspartic acid 64	37%	46%
3. Carbohydrate		
N-linked glycans		
Asn 157 }	High heterogeneity	Low heterogeneity
Asn 167 }	fully sialylated	<fully sialylated
O-linked glycans		
Ser 53	$(Xyl)_{1-2}$-Glc	$(Xyl)_2$-Glc
Ser 61	NeuAcGalGlcNAcFuc	NeuAcGalGlcNAcFuc
Thr 159, 169 & 172	Classical, partially filled	Classical, partially filled
4. Tyr 155 sulphation	>90%	<15%
5. Ser 158 phosphorylation	>90%	<1%

Footnote: Adapted from ref. [37] with permission. *The 10 completely modified Gla residues occur at amino acid residues 7, 8, 15, 17, 20, 21, 26, 27, 30 and 33. Gla residues at positions 36 and 40 are incompletely modified, as indicated, in recombinant factor IX.

ent in the extent of N- and O-linked carbohydrate side chains, and in sulphation and phosphorylation levels of particular residues (Tab. 1). The extent of β-hydroxyaspartate modification at serine 64, however, was similar. Fortunately, none of the differences in post-translational modification seemed to affect the biological activity of factor IX, although the differences in sulphation and phosphorylation, it was suggested, may explain the slightly lower recovery (on average about 20% lower) of recombinant, as compared to blood-derived, factor IX seen in patients [37].

In summary, recombinant factor IX is already really a "second-generation" recombinant product because it is prepared from cultured cells and purified under conditions where there is no contact with human or bovine proteins [37]. Thus the product should be free of known blood-borne viral diseases, such as HIV, hepatitis A, B or C and parvoviruses. There should also be no risk from diseases caused by prions, such as Creutzfeldt-Jakob disease (CJD) or the variant CJD, caused by the prion of bovine spongiform encephalitis in cattle. Recombinant factor IX (BeneflX) was approved for sale in the USA and Canada in 1997 and in Europe in 1998.

Recombinant factor VIII

When factor VIII was first cloned and expressed in cultured cells by two competing companies in 1984, this achievement was hailed as one of the most exciting advances to be reported by the biotechnology industry [39]. However, in a Nature editorial in the same week that the factor VIII cloning was announced, we were poignantly reminded that the first deaths had occurred in Australia and in the UK from HIV-contaminated blood. Clearly blood-derived clotting factors were potentially dangerous and would eventually be superseded by recombinant proteins. Not for the first time was the fear of disease driving scientists and biotechnology companies to find improved and safer medicines.

Cloning and expression of factor VIII

The factor VIII gene and its cDNA were cloned by the use of oligonucleotide probes based on the amino acid sequence of peptides isolated from either human or porcine factor VIII, essentially by similar methods to those employed previously to clone factor IX. Due to its low concentrations in plasma, its high molecular weight and extreme sensitivity to proteolytic processing, it was a very difficult protein to purify. The breakthrough came when amino acid sequence information on peptide fragments of human or porcine factor VIII became available [40, 41].

Surprisingly, a unique 36-nt long oligonucleotide, rather than a mixture of oligonucleotides, was successfully used as a probe in one report [40], and in the other [41] two sets of mixed oligonucleotides, either 45-nt long or 15-nt long, were used. Because of uncertainty as to whether factor VIII was synthe-

sized in the liver, both groups initially cloned the factor VIII gene by screening genomic libraries of clones in bacteriophage λ with these probes. The gene turned out to be 180 kb long with 26 exons and at that time was the longest known gene. Human cDNA clones were then subsequently isolated, using genomic probes, by screening appropriate human cDNA libraries. Full-length cDNAs were then used for expression studies by introducing expression plasmids with heterologous viral promoters into BHK [40] or monkey kidney (COS-1) cell lines [41]. Human factor VIII was secreted into the medium of these transfected cells and shown to be active in a clotting assay. Cloning and characterization of the factor VIII gene and its cDNA had been a long task, but its expression was initially surprisingly straightforward.

Development of recombinant factor VIII for clinical use

The subsequent production and purification of recombinant factor VIII from cultured mammalian cells by the biotechnology industry were quite rapid and commensurate with the urgency in producing recombinant factor VIII, which was free of viral contamination for the treatment of haemophilia A patients.

Initially, however, there was some difficulty in the approach developed by Kaufman's group at Genetics Institute in obtaining high yields of factor VIII secreted by CHO cells. Factor VIII cDNA had been introduced into CHO cells by linking it to the selectable, amplifiable marker, dihydrofolate reductase (DHFR). Protein expression levels were, however, much lower than had been observed with other cDNAs, e.g., factor IX, in CHO cells. It emerged that the reason for this was that during intracellular processing of factor VIII in the ER, a significant proportion of the factor VIII became bound to the chaperone, immunoglubulin-binding protein (BiP), through a primary binding site of its A1-domain. Interactions also occurred between factor VIII and 2 other protein chaperones, calnexin and calreticulin, primarily mediated by the B-domain of factor VIII. It was suggested that factor VIII has unique requirements, not shared by the related coagulation factor V, for carbohydrate processing and molecular chaperone interactions that may limit its secretion [42]. In practice the problem of the low yield of secreted factor VIII was initially overcome, at least in part, by the addition of von Willebrand factor (vWF) (the cofactor with which factor VIII is normally associated as a high-molecular-weight complex in plasma) to the tissue culture medium. Thus factor VIII secreted in the absence of vWF appeared to be rapidly degraded. In its presence, however, factor VIII associated with vWF to form high-molecular-weight multimers. This stabilized the factor VIII, protecting it from proteolytic degradation and improving factor VIII yields significantly [43].

Finally, in order to develop a large-scale process for production of factor VIII, the vWF cDNA was introduced into the factor VIII-expressing CHO cells by linking it to a second amplifiable marker, the adenosine deaminase gene. Co-expression of factor VIII and vWF was thereby achieved even in serum-free medium (Fig. 4), improving the factor VIII yields by 1–2 orders of magnitude [44, 45]. Even so, the yield of factor VIII was still significantly lower

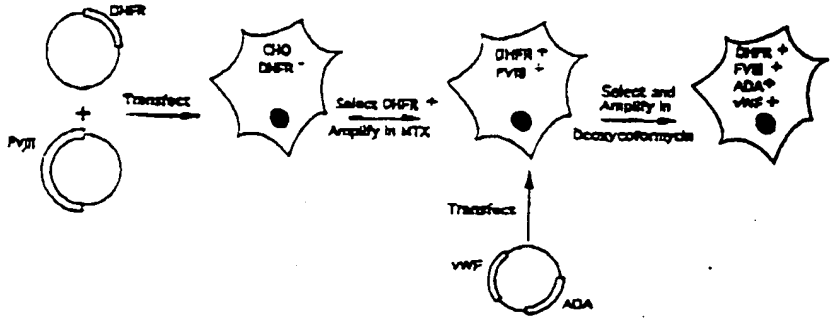

Figure 4. Derivation of factor VIII and von Willebrand factor coexpressing CHO cells used for the manufacture of recombinant factor IX. Adapted from Kaufman [45] with permission. Plasmids encoding DHFR and factor VIII were transfected into DHFR-deficient CHO cells and transformants selected by growth in nucleoside-free medium. Cells were then selected in increasing concentrations of methotrexate to obtain a cell line that had amplified the DHFR and factor VIII genes. These cells were subsequently transfected with a vWF expression plasmid containing an ADA (adenosine deaminase) gene and selected for growth in the presence of cytotoxic concentrations of adenosine with increasing concentrations of 2'-deoxycoformycin. The final cell expressed DHFR, ADA, vWF and factor VIII.

than that of the co-expressed vWF. This suggested that the intracellular regulation of factor VIII mRNA levels and/or regulation of the intracellular folding and processing of factor VIII were still limiting the yields of factor VIII secreted into the culture medium [44]. The factor VIII-expressing CHO cell line is cultured in a defined, serum-free medium in 2500 l bioreactors for the large-scale manufacture of recombinant factor VIII. However, it should be noted that the culture medium is reported to contain (unspecified) added bovine proteins from natural sources, which were, however, tested to minimize the risk of introducing viral contamination [46].

By contrast, a different cell line was used for factor VIII expression in the approach adopted by Genentech/Bayer. A clone of BHK cells was isolated after introducing the factor VIII cDNA, linked to DHFR, into chromosomal DNA followed by amplification of the factor VIII copy number by selection with methotrexate. The BHK clone used for the commercial production of factor VIII contains about 150 copies of the factor VIII cDNA per cell [47]. For the subsequent manufacture of factor VIII in deep-tank stirred suspension fermenters, a proprietary (unspecified), serum-free medium was used [48]. This medium presumably contained some added protein, since there was no requirement to introduce the vWF cDNA to improve the stability of the factor VIII secreted in this BHK cell line, unlike the situation in CHO cells (see above).

Both recombinant factor VIII products (Genetics Institute or Genentech), after purification by immuno-affinity chromatography with monoclonal antibodies, ion-exchange chromatography and other methods, were reported to be essentially indistinguishable from plasma-derived factor VIII [45, 48]. The full-length recombinant factor VIII produced by both companies may be

regarded as a "first-generation" product, since the CHO cells used to produce factor VIII were grown in a tissue culture medium that contained protein additives. Moreover, the resultant purified factor VIII was stabilized by the addition of human albumin. Thus there remains a small theoretical risk, since natural products from humans or cattle were used in their manufacture, that heat-resistant viruses, such as B19 parvovirus (causing fifth disease) or, more seriously, prions, might still be present. Approval was granted in 1992 for the sale, in the USA, of Recombinate (manufactured by Genetics Institute/Baxter) and in 1993 for the sale of Kogenate (manufactured by Genentech/Bayer, originally Miles/Cutter).

B-domainless recombinant factor VIII

A second generation, B-domainless factor VIII, recombinant factor VIII SQ ("Refacto": Pharmacia/Upjohn/Genetics Institute), is now (1999) also approved [49]. Early work, both by recombinant DNA and classic protein chemistry methods, had shown that factor VIII with its B domain deleted was active in clotting assays [50–52]. Factor VIII SQ was developed from one particular construct, which retained the factor VIII processing protease cleavage sites at amino acids 740 and 1649 with only 14 residues of the B domain still present. This was almost fully processed when expressed in CHO cells, consistent with its retaining a furin cleavage site preceding amino acid 1649 [53]. An advantage of factor VIII SQ, in comparison to the first-generation full-length factor VIII products, is that it is formulated without the addition of human albumin. However, human albumin is still added to the tissue culture medium in which the CHO-expression construct is grown [49]. Thus, these second-generation products have a reduced theoretical risk of transmission of human viruses [49].

Clinical experience with recombinant factors

Recombinant products offer the greatest margin of safety for haemophilic patients and have been recommended for the treatment of all patients. However, they are significantly more expensive and many patients continue to receive plasma-derived products. In addition to offering an increased margin of safety with regard to viral contamination, recombinant products also offer a solution to the problem of the burgeoning demand for coagulation factors which continues to rise steeply. Thus 62 million units of factor VIII were used in the UK in 1981, but by 1998 consumption had increased to 200 million units. This largely reflects the changing pattern of treatment, with patients now being encouraged to treat themselves at home on a prophylactic basis to prevent joint bleeds. The plasma half-life and recovery of the various recombinant factor VIII products are identical to those observed with conventional plasma-derived products and are typically around 14–16 hours. In contrast to many plasma-derived products, none of the recombinant products contain vWF, and

thus they are of no use in the treatment of this different congenital haemorrhagic disorder. The plasma half-lives of recombinant factor IX are approximately 18 hours, which are also the same as those observed with plasma-derived factor IX products [37].

Recombinant factor VIIa

Recombinant factor VIIa (Novoseven, Novo Nordisk) has proved very useful in the clinical management of patients with either haemophilia A or B and inhibitory antibodies, as well as those with acquired haemophilia. It by-passes the requirement for either factor VIIIa or IXa because it activates factor X (see Fig. 1). Control of bleeding episodes in patients with inhibitory antibodies is a major clinical challenge. Most of the clinical experience relating to use of recombinant factor VIIa has been gained outside the setting of formal clinical trials, and this has hindered licensing in the United States [54]. The product is extremely expensive and this is likely to hinder widespread use when other products may be just as effective. It is valuable in patients who have high titres of inhibitory antibodies. It is of particular value, however, in the few patients with haemophilia B and inhibitory antibodies, as administration of either plasma-derived or recombinant factor IX in these cases can result in serious anaphylactic reactions [55]. Factor VIIa has a plasma half-life of only approximately two hours, so that frequent bolus injections are required to control bleeding; it is not licensed for continuous intravenous administration. No specific adverse effects have been identified. The excellent safety profile, including lack of allergic reactions, has encouraged some clinicians to employ this product for first-line treatment of bleeds at home [56]. In the past, physicians were often reluctant to undertake elective surgery in haemophiliacs with inhibitors, but such procedures can now be carried out with confidence with factor VIIa [57].

Safety issues

Concerns about recombinant coagulation factor concentrates have focused on three main issues: viral safety, incidence of inhibitor development and problems with laboratory assays. It is somewhat ironic that several currently licensed preparations of recombinant factor VIII actually contain a considerable quantity of human, plasma-derived albumin that is added to the product as a stabilizer. Furthermore, most recombinant products are not subjected to specific virucidal treatment, such as heat treatment, during manufacture. Alternative stabilizers have been developed and clinical trials with these second-generation recombinant factor VIII products are already underway. Recombinant factor IX does not require the addition of albumin as a stabilizer. Bovine proteins are incorporated in growth media used in the manufacture

Table 2. Recombinant factor concentrates

Product and manufacturer	Viral inactivation	Human albumin	Bovine protein
Recombinate (Baxter)	No	Yes	Yes
Kogenate[1] (Bayer)	Yes	No[2]	No
Refacto (Wyeth)	Yes	No[2]	No
BeneFIX (Baxter)	Yes	No	No
Novo Seven (Novo)	No	No	Yes

Footnotes: [1] identical to Helixate (Aventis); [2] although cells are grown in human albumin

of some products (see above). The current status of the various recombinant products is summarized in Table 2.

Concern has also been expressed that the use of highly purified factors might result in a greater incidence of inhibitory antibodies. Unfortunately, it is not possible to give a definitive answer to this important issue, as no prospective, double-blinded clinical studies have been conducted in which the incidence of inhibitor development has been compared in patients receiving recombinant products and those receiving conventional plasma-derived products. Approximately 5% of patients with haemophilia A in the UK are known to have developed inhibitory antibodies (or about 15% if severe patients with a baseline of <2% factor VIII are considered). The first two clinical trials of two different recombinant products in previously untreated patients (PUPs) reported an incidence of inhibitor development of around 20% [58, 59]. Inhibitors appeared after a mean of 9 exposure days in both clinical trials. A more recent study from France with a longer follow-up period reported an inhibitor incidence of 28% in patients receiving recombinant factor VIII, in contrast to an incidence of 9% in a historical control group which received only plasma-derived products [60]. Similar results have been observed in other groups. Most of the cases involved low titre and/or transient inhibitors that disappeared as treatment was continued. It is quite probable that many cases of transient or low-titre inhibitors amongst patients receiving conventional concentrates were simply not identified in the past, and most haematologists do not believe that the incidence of significant inhibitors in patients receiving recombinant products is truly elevated. The median number of exposure days until detection of the inhibitor was 9.5 days (range 5–14 days).

By contrast with recombinant factor VIII, there is no evidence of an increased risk of inhibitor development with the use of recombinant factor IX. In clinical trials, only one of 31 PUPs developed an inhibitor and only one of 56 previously treated patients (PTPs) developed a transient inhibitor. These findings are comparable with the incidence of inhibitors of approximately 1% of historical controls with haemophilia B. The formation of specific antibodies against recombinant factor VIIa has not been identified in subjects with either congenital or acquired haemophilia.

The ability to assay the activity of factor VIII both in actual coagulation factor concentrates and in the plasma of haemophiliac patients after infusion of products is vital for clinical care. Various methods have been developed for the assay of factor VIII, including the one-stage, two-stage and automated chromogenic methods. In general, clinical laboratories tend to prefer the one-stage method, which is both faster and easier to perform, for assay of factor VIII in both concentrates and patient plasma. By contrast, the potency of concentrates is now assigned by the chromogenic method, which is actually based on the two-stage method. Traditionally, human plasma is used as the standard for assays of plasma factor VIII. When plasma levels of factor VIII are assayed after infusion of recombinant factor VIII, recovery is significantly greater when using the chromogenic assay compared to the conventional one-stage assay. Pharmacokinetic evaluation also shows a 25% greater AUC (Area under curve (concentration × time) with the chromogenic method, but half-lives are identical on calculations using either assay method. It has recently been shown that these differences may be resolved by using recombinant concentrate diluted in haemophilic plasma as the laboratory standard. The recovery of recombinant factor IX in patients is slightly lower (on average by about 20%) than that observed with standard, plasma-derived products (see above). This means that a higher dose has to be given to patients to achieve a defined target plasma-level. Marked differences in laboratory assay results have been observed with "Refacto" (B-domainless factor VIII). The potency of this product should be assayed using the chromogenic substrate method, since conventional one-stage assays usually underestimate the real potency.

Summary and prospects

Recombinant factors VIII, IX and VIIa are now available for treatment of haemophilia A & B patients in countries where this newer form of "protein therapy" is affordable. They are safer than plasma-derived factors because they carry no, or minimal, risk of contamination by viruses. Unfortunately, at present, they are more expensive. In the future, gene therapy for haemophiliacs may become available. This subject has certainly been widely discussed as a potential cure for patients and there is considerable current interest in this topic [61]. Good progress has been made with adenoviral and adeno-associated viral vectors and clinical trials are in progress with adeno-associated viral vectors. Thus gene therapy is some way off and may not become available for haemophiliacs for some time.

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EXHIBIT C

TRANSFUSION

Revised: November 1999

Descriptions, Indications, and Therapeutic Effects
Factor Concentrates

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Factor VIII Concentrates

Description

Factor VIII concentrates are a commercially prepared, lyophilized powder purified from human plasma to treat patients with hemophilia A or von Willebrand's disease. Alternatively, recombinant (synthetic) protein is purified from genetically engineered non-human cells grown in tissue culture. The quantity of factor VIII coagulant activity is stated on the bottle. One factor VIII concentrate unit equals the clotting activity in 1 ml of fresh plasma. Factor VIII concentrate is cell free and is administered without regard to patient or donor ABO or Rh type. It is heat treated and/or solvent detergent treated to reduce the risk of virus transmission. Current processes appear to have eliminated the risk of HIV, HBV and HCV transmission. Concentrates differ in the purification procedures. Highly purified factor VIII, e.g. preparations purified over a monoclonal antibody column or current recombinant factor VIII concentrates, are stabilized by adding 98% of pasteurized human albumin. Porcine factor VIII concentrate is available for patients with high titer anti-human factor VIII allo or autoantibody inhibitors. Factor VIII concentrates are stored refrigerated at 2-8°C for up to two years from the date of manufacture (expiration date will be indicated on each vial). Some preparations may be kept at room temperature for extended periods. Once reconstituted, it should not be refrigerated. Factor VIII concentrate should be infused within 4 hours of preparation to reduce the risk of bacterial growth. Vials are usually shipped to a hospital pharmacy, blood service or nursing unit and mixed there prior to use. Many patients or families receive them directly for home care.

Indication

Factor VIII concentrate is indicated for the treatment of bleeding or imminent invasive procedures in patients with hemophilia A (congenital factor VIII deficiency) and for patients with low titer factor VIII inhibitors. Regular prophylactic doses are sometimes used as well as daily doses in some hemophilic inhibitor patients to try to induce immune tolerance. Patients with von Willebrand's disease respond to one specific, pasteurized intermediate-purity concentrate in which von Willebrand factor activity is relatively preserved.

Therapeutic Effect

Dosage is dependent on the nature of the injury, the degree of factor deficiency, the weight of the patient and the presence and level or absence of factor VIII inhibitors. The half life of circulating factor VIII is 8 to 12 hours, therefore transfusions may need to be repeated every 12 to 24 hours to maintain hemostatic levels. Following surgery, it is necessary to maintain hemostatic levels for up to two weeks to prevent delayed bleeding and promote wound healing in the hemophilic patient. The Puget Sound Blood Center's Hemophilia Program is available for consultation at 206-292-6507 (or 1-800-552-0640) or 206-292-6525 (and page) on evenings or weekends.

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EXHIBIT D

Emory Health Sciences Press Release

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October 10, 2000

EMORY'S COMPREHENSIVE HEMOPHILIA PROGRAM IS A LEADER IN CLINICAL RESEARCH AND ADVANCED TREATMENTS FOR ADULTS AND CHILDREN

The comprehensive hemophilia program at Emory University has become a national leader in research and treatment programs that are helping improve the lives of the 15,000 hemophiliacs living in the U.S. Although these patients now can enjoy a normal life span, they must rely on infusions of blood products to treat frequent bleeding episodes, then cope with resulting complications, including inhibitors that render the products ineffective and the threat of infections and joint diseases caused by internal bleeding. In the past, the cure for hemophilia has sometimes been worse than the disease.

Emory hematologists participate in a number of clinical trials to improve treatments for hemophilia patients, including a recently concluded international study sponsored by Bayer to test a new formulation of blood clotting products. The new formula, called Kogenate FS, is almost completely free of any human or animal components and should make hemophiliacs feel safer than ever about using genetically engineered clotting factors. Kogenate FS has recently been approved by the Food and Drug Administration.

Individuals with hemophilia are missing the gene that makes Factor VIII, a critical part of the blood clotting machinery. Factor VIII is a component of fibrin, which is the cement the body uses to seal a wound after platelets first plug it up, much like the Dutch boy putting his finger in the dike while awaiting help.

In the past, Factor VIII products to treat hemophilia patients were made by concentrating clotting factor gathered from the plasma of a large group of donors. In the mid 1980s, when scientists discovered that these blood products could transmit diseases like HIV and hepatitis C, they began heating Factor VIII products to kill these viruses.

In the early 1990s, scientists carried safety one step further with genetically engineered recombinant Factor VIII products made by inserting the factor VIII gene into a cell line and producing mass quantities of concentrated human factor VIII. Although these products contained no human or animal products, they were stabilized with a small amount of albumin, a human blood component. Kogenate FS -- the newest FDA-approved product -- uses small amounts of albumin in the initial "fermenting" solution, but in the final stage, albumin is removed, leaving the product almost completely free of any human or animal components. The Factor VIII is then stabilized with sucrose. The product is said to be sucrose-formulated and albumin-free in final formulation.

"Our research found that the new product works just as well as the current products and appears to offer a greater safety margin against infectious agents," says Thomas Abshire, M.D., medical director of Emory's hemophilia program and one of the principal investigators for the study.

Emory has just completed another randomized study, in collaboration with Schering Plough, Inc. and the American Red Cross, in which hemophilia patients with hepatitis C were treated either with a combination of interferon and Ribavirin, or with interferon alone, which is the

known treatment. Preliminary results presented at the World Federation of Hemophilia in July determined that the combination therapy is better than interferon alone.

Emory also is a world leader in treating joint disease in hemophiliacs -- a common problem caused by bleeding into joints, which causes irritation in the lining of the joint cavity and creates a cycle of bleeding and inflammation. Surgeons and hematologists have collaborated on a study of arthroscopic synovectomy, in which a small endoscope is inserted into the ankle, elbow, or knee to clean out the thickened lining. When surgery is not an option, physicians use an alternative technique called radionuclide synovectomy to inject a radioisotope into the joint that eliminates the abnormal lining. Drs. Michael Busch and Amy Dunn coordinate this program.

Emory's comprehensive adult and pediatric hemophilia program includes hematologists, infectious disease specialists, hepatologists, orthopaedic surgeons, physical therapists and specialty nurses. The program receives some federal funding through the Maternal and Child Health Bureau (MCHB) and the Centers for Disease Control and Prevention (CDC). Two adult and two pediatric hematologists treat 350 patients, including 140 children. The staff also works closely with a program at Children's Healthcare of Atlanta at Scottish Rite, which treats 150 additional children. Dr. Abshire also is Medical Director for MCHB Region IV South hemophilia programs that include Alabama, Mississippi, Georgia and Florida.

Although the number of hemophilia patients is small relative to many other diseases, it commands a great deal of attention because it is so expensive to treat. Clotting factor for a mild joint bleed in a typical 7-year-old child, even at reduced rates, averages $600 per infusion, with some patients needing several infusions per week. "There is a motivation to produce a better product and one you can use less of, which may come with the eventual development of gene therapy for Factor VIII," says Dr. Abshire.

Other current clinical trials at Emory aimed at treating bleeding disorders and their complications include: (1) a study of children who experience clotting problems from permanent IVs; (2) a CDC-sponsored study designed to identify and treat women with undiagnosed bleeding disorders who are experiencing abnormal bleeding with menstrual periods (hematologist Sidney Stein, M.D., leads the Emory component of this multi-site study); (3) multiple clinical trials designed to evaluate the safety and efficacy of new products used to treat bleeding episodes; (4) multiple AIDS Clinical Trials Group (ACTG) studies for our patients that contracted HIV infection from blood products prior to the development of safer products.

The Emory hemophilia treatment center also participates in studies designed to identify the safest and most cost effective methods for preventing the complications of congenital bleeding disorders, including the CDC-sponsored Universal Data Collection System and the National Hemophilia Foundation's National Prevention and Awareness Campaign.

Exciting research advances are on the horizon to deal with the problems faced by the 20 percent of hemophilia patients who have antibodies that inhibit the effectiveness of substitute Factor VIII products. Emory hematologist J.S. "Pete" Lollar, M.D., is conducting groundbreaking research in the laboratory that includes gene therapy and an improved Factor VIII molecule constructed from a combination of human and pig Factor VIII genes.

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Last Updated: October 13, 2000

EXHIBIT E

News

January 10, 2001

Refacto (rFVIII) to Become Available

Wyeth/Genetics Institute has announced that their second-generation recombinant factor VIII product, ReFacto will be available in the United States beginning on January 29, 2001. ReFacto is the first recombinant factor VIII product formulated without human serum albumin in its final formulation.

ReFacto is indicated for the control and prevention of hemorrhagic episodes and for surgical prophylaxis in patients with hemophilia A. ReFacto is also indicated for short-term routine prophylaxis to reduce the frequency of spontaneous bleeding episodes.

Wyeth/Genetics Institute claims that dosing for ReFacto will be exactly the same as the other second-generation recombinant factor VIII products currently on the market. Further, Wyeth/Genetics Institute has announced that 2000 I.U. sizes for both ReFacto and their recombinant factor IX, BeneFix, will be available.

Due to limited supply of ReFacto however, Wyeth/Genetics Institute will be placing limits on individual customer size orders. Wyeth/Genetics Institute has devised an allocation method whereas the 18 largest homecare companies and the hemophilia treatment centers will be allocated product based on the percentage of hemophilia A patients they see. For example, if ABC Homecare has a hemophilia A base of 10% of the overall hemophilia A population, they would be allocated 10% of Wyeth/Genetic Institute's inventory of ReFacto.

Increased production for ReFacto is expected sometime next year as Wyeth/Genetics Institute is working towards completion of a refurbished manufacturing plant in St. Louis, Missouri. Currently, product is being imported from a manufacturing facility in Stockholm, Sweden. The St. Louis facility is forecast for completion in early 2002.

The list price for ReFacto will be $1.09. However, Wyeth/Genetics Institute is going to offer a bulk contract price of $0.84. The list price for other second-generation recombinant factor VIII products are similar (Kogenate FS - $1.13; Helixate FS - $1.11).

The Average Wholesale Price (AWP) of ReFacto is expected to be the similar to other second-generation recombinant factor VIII's as well. Wyeth/Genetics Institute expects the

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AWP of ReFacto to be the list price ($1.09) multiplied by 25%. It this is the case, ReFacto's AWP would be $1.36 whereas Kogenate FS would be $1.41 and Helixate FS $1.38.

Wyeth/Genetics Institute is also working on a third-generation recombinant factor VIII named ReFacto AF (Albumin Free). The St. Louis manufacturing facility has been selected to product ReFacto AF, once the FDA approves that product. Wyeth/Genetics Institute has yet to decide if they will phase out ReFacto once ReFacto AF has been approved. In similar situations, Bayer chose to phase out their first generation recombinant product, Kogenate, once their second-generation product, Kogenate FS was licensed. However, Baxter Hyland Immuno has decided to keep their first generation recombinant product Recombinate on the market, even after their third-generation recombinant product is licensed.

Wyeth/Genetics Institute currently manufactures and distributes ReFacto in Europe. Moreover, they are currently the only manufacturers of a recombinant factor IX product, BeneFix.

EXHIBIT F

US006171825B1

(12) **United States Patent**
Chan et al.

(10) **Patent No.: US 6,171,825 B1**
(45) **Date of Patent: Jan. 9, 2001**

(54) **PREPARATION OF RECOMBINANT FACTOR VIII IN A PROTEIN FREE MEDIUM**

(75) Inventors: **Sham-Yuen Chan**, El Sobrante; **Kathleen Harris**, Oakland, both of CA (US)

(73) Assignee: **Bayer Corporation**, Berkeley, CA (US)

(*) Notice: Under 35 U.S.C. 154(b), the term of this patent shall be extended for 0 days.

(21) Appl. No.: **09/146,708**

(22) Filed: **Sep. 4, 1998**

Related U.S. Application Data

(63) Continuation-in-part of application No. 08/844,714, filed on Apr. 18, 1997, now Pat. No. 5,804,420.

(51) **Int. Cl.**[7] **C12P 21/04**; C12N 5/00; A61K 35/14; C07K 14/00

(52) **U.S. Cl.** **435/69.6**; 435/383; 435/404; 435/406; 530/383

(58) **Field of Search** 435/69.6, 172.3, 435/325, 352, 363, 373, 383, 404, 406; 530/383

(56) **References Cited**

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WO98/15614 * 4/1998 (WO) 435/325

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Primary Examiner—Karen Cochrane Carlson
Assistant Examiner—Holly Schnizer
(74) *Attorney, Agent, or Firm*—James A. Giblin

(57) **ABSTRACT**

Recombinant Factor VIII can be produced in relatively large quantities on a continuous basis from mammalian cells in the absence of any animal-derived proteins such as albumin by culturing the cells in a protein free medium supplemented with polyol copolymers, preferably in the presence of trace metals such as copper. In very preferred embodiments, the medium includes a polyglycol known as Pluronic F-68, copper sulfate, ferrous sulfate/EDTA complex, and salts of trace metals such as manganese, molybdenum, silicon, lithium and chromium. With an alternative medium which included trace copper ions alone (without polyol copolymers) we were also able to enhance the productivity of Factor VIII in recombinant cells such as BHK cells that are genetically engineered to express Factor VIII.

11 Claims, No Drawings

PREPARATION OF RECOMBINANT FACTOR VIII IN A PROTEIN FREE MEDIUM

This is a continuation-in-part of prior application Ser. No. 08/844,714 filed Apr. 18, 1997 now U.S. Pat. No. 5,804,420.

BACKGROUND OF THE INVENTION

1. Field

This disclosure is concerned generally with the manufacture of recombinant Factor VIII and specifically with the manufacture of recombinant Factor VIII in a serum or protein free medium.

2. Prior Art

Hemophilia A is an X-linked recessive genetic disorder that is due to a defective or deficient Factor VIII molecule, resulting in a hemorrhagic tendency. To control bleeding episodes, hemophiliacs are treated with Factor VIII. Historically Factor VIII has been isolated from human blood plasma. However, therapy with plasma-derived Factor VIII has been associated with transmission of several human viruses, such as hepatitis and human immunodeficiency viruses.

With the advent of recombinant DNA technology, the structure of human Factor VIII and its gene has been elucidated. The transcription product of the gene, which is derived from 26 exons, is a messenger RNA molecule of ~9000 bases in length, coding for a large protein of 2351 amino acids. Structural studies of Factor VIII indicate that it is a glycoprotein containing a significant number of carbohydrate residues.

The cDNA coding for Factor VIII has been cloned and stably expressed in baby hamster kidney (BHK-21) and Chinese hamster ovary (CHO) cells. Commercial processes have been developed to produce recombinant Factor VIII for treatment of hemophilia A.

Recombinant Factor VIII is currently manufactured by genetically engineered mammalian cells, thus obviating the reliance on plasma and minimizing any possible risk of virus transmission.

Gene amplification has been the method of choice to derive high production cell lines for therapeutic proteins. The amplification strategy involves the linking of a transcriptional unit encoding the desired protein to an amplifiable marker such as dihydrofolate reductase. Transfection techniques are then applied to transfer the vector DNA to recipient cells. Cell populations are selected for increased resistance to the drug of choice such as methotrexate. The establishment of a stable cell clone is accomplished by limiting dilution cloning. These cell clones are then adapted to a serum-free production medium and monitored for production of the desired protein.

For labile proteins such as Factor VIII, human albumin has been added as a stabilizer during the preparation and purification procedures. Although the albumin is subjected to a viral inactivation step by pasteurization, it would be ideal if recombinant Factor VIII could be manufactured in the complete absence of human and animal blood proteins. I have now found this is possible by using novel cell culture media. Details are described below.

SUMMARY OF INVENTION

The method for the continuous production of relatively large quantities of recombinant Factor VIII (rFVIII) from mammalian cells in the absence of any human or animal-derived plasma proteins comprises culturing the mammalian host cells in a protein-free medium supplemented with a polyol polymer such as Pluronic F-68 and copper ions. The preferred medium includes copper sulfate, a ferrous sulfate/EDTA complex, and the salts of trace metals such as manganese, molybdenum, silicon, lithium, and chromium. Alternatively we have also found that addition of copper ions alone (without polyol polymers) in the protein free medium may be used to enhance productivity of rFVIII in recombinant mammalian host cells, as described below under Additional Studies.

DETAILED DESCRIPTION OF THE INVENTION

Recent advances in recombinant protein expression technology have made possible the production of protein in large quantities in mammalian cells. Host cells suitable for Factor VIII production include cell lines such as baby hamster kidney (BHK) cells, Chinese hamster ovary (CHO) cells, and human embryonic kidney (HEK) cells. Particularly preferred are baby hamster kidney cells, specifically those transfected with a gene capable of directing the expression of Factor VIII as described in Wood et al. (1984) (including derivatives such as clonal variants and progeny thereof). Such a cell line has been deposited with the American Type Culture Collection and has been assigned the accession number ATCC CRL-8544.

The desired host cell line carrying the Factor VIII gene is typically adapted to grow as suspension cultures in a protein-free production medium which is supplemented with lipoprotein. The basal medium chosen for culturing the host cell line is not critical to the present invention and may be any one of, or combination of those known to the art which are suitable for culturing mammalian cells. Media such as Dulbecco's Modified Eagle Medium, Ham's Medium F-12, Eagle's Minimal Essential Medium, and RPMI-1640 Medium, and the like, are commercially available. The addition of growth factors such as recombinant insulin is conventional in the art.

Due to the labile nature of Factor VIII, the productivity of the engineered host cells is severely reduced under protein-free conditions. Human serum albumin is commonly used as a serum-free culture supplement for the production of recombinant proteins. Human serum albumin serves many functions including: (1) as a carrier for fatty acids, cholesterol and lipophilic vitamins, steroid hormones and growth factors; (2) as a protective agent against damages due to shear forces; (3) as a buffer for pH changes; and (4) as an osmotic pressure regulator. Another critical role of albumin is perhaps to protect labile proteins such as Factor VIII from proteolysis by serving as a substrate for proteases.

The impurities present in albumin preparations may also contribute to the stabilizing effect of albumin. Factors such as lipoprotein (Chan, 1996) have been identified as a replacement for human serum albumin for the production of recombinant Factor VIII under serum-free conditions.

Our attempt to develop a production medium free of human plasma-derived albumin led to the inventions of this disclosure, basal protein-free media for recombinant Factor VIII production. The preferred medium consists of modified Dulbecco's Minimum Essential Medium and Ham's F-12 Medium (50:50, by weight) supplemented with recombinant insulin (Nucellin, Eli Lilly) at 10 μg/ml, and $FeSO_4$•EDTA (50 μM). With the exception of Factor VIII production, engineered BHK cells grow well in this protein-free basal medium.

Surprisingly, the addition of a polyol such as Pluronic F-68 had no effect on growth but enhanced the specific productivity of the BHK cells for Factor VIII. Serendipitously, the addition of copper sulfate further enhances the production of Factor VIII. Also the inclusion of a panel of trace metals such as manganese, molybdenum, silicon, lithium, and chromium lead to further increases in Factor VIII production. A continuous process was then developed for Factor VIII production under human plasma-derived protein-free conditions. Further information regarding the use of Pluronic polyols can be found in Papoutsakis (1991) and Schmolka (1977).

Pluronic F-68, a polyglycol, (BASF, Wyandot) is commonly used to prevent foaming that occurs in stirred cultures, and to protect cells from shear stress and bubble damage in sparged cultures. Pluronic F-68 is a nonionic block copolymer with an average molecular weight of 8400, consisting of a center block of poly(oxypropylene) (20% by weight) and blocks of poly(oxyethylene) at both ends. Extensive research on the role of Pluronic F-68 indicates that Pluronic F-68 acts as a surfactant and prevents damage to cells by allowing the drainage of cells away from bubbles formed in the bioreactors during stirring or sparging. However, several investigators have noticed beneficial effects of Pluronic F-68 on growth under culture conditions in which shear is minimal (Mizrahi, 1975; Murhammer and Goochee, 1990). Co-purification of lipids with Pluronic F-68 during product purification provides anecdotal evidence that the Pluronic polymer may substitute for albumin not only as a surfactant, but may also act as a carrier for lipids. Pluronic F-68 may also prevent membrane damage from killing cells before repair can be effected, possibly by direct intercalation into the membrane. The role of Pluronic F-68 in acting as a metal ions buffer is completely unknown.

Although there are reports that Pluronic F-68 in media can increase volumetric productivity, the mechanism of action appears to be maintenance of cell viability (Schneider, 1989; Qi, 1996). To our knowledge, this is the first time that Pluronic F-68 has been seen to increase specific production of a particular protein product. Since viabilities and growth rates are comparable in our system with and without Pluronic F-68, maintenance of cell viability cannot be the mechanism of action of Pluronic F-68 in our system. However, the effect of Pluronic F-68 addition is immediate and dramatic, whatever the mechanism.

It is anticipated that a range of other polyols would have similar effects. Such other polyols include nonionic block copolymers of poly(oxyethylene) and poly(oxypropylene) having molecular weights ranging from about 1000 to about 16,000.

In addition to conventional suspension culturing techniques such as shake flasks, spinner flasks, and roller bottles, the method of the present invention is also applicable for use with perfusion and batch bioreactors. Following culturing the host cells, the Factor VIII may be recovered from the spent medium by standard methodologies such as ultrafiltration or centrifugation. If desired, the recovered Factor VIII may be purified by, for example, ion exchange or size exclusion chromatography, immuno-affinity or metal chelate chromatography, and the like.

As used herein, a "human or animal protein-free medium" is a cell culture medium which is free of any protein that has been derived from a human source or an animal source. Proteins which are isolated from human or animal sources (such as plasma) inherently carry the risk of introducing viral contamination. The goal of a human or animal protein-free medium is thus to eliminate or at least greatly reduce the risk of viral transmission.

EXAMPLE 1

Baby hamster kidney (BHK-21) cells transfected with a gene capable of directing the expression of Factor VIII were obtained from Genentech, Inc., South San Francisco, Calif., U.S.A. The cell line was prepared as described in detail in Wood et al. (1984) and was deposited with the American Type Culture Collection and given accession number ATCC CRL-8544. A clonal variant of this cell line was also obtained from Genentech, Inc., and used in all examples.

The BHK-21 cells containing the gene encoding Factor VIII were cultivated as suspension cultures in shake flasks using a serum-free basal medium containing the following: Ham's F-12 Medium and Dulbecco's Minimal Essential Medium (50:50, by weight), Nucellin (recombinant insulin, 5–10 μg/ml), $FeSO_4$•EDTA (50 μM), and $MgCl_2$ (15 mM). Cells were maintained and passaged at 48 hour intervals. Cells were spun down at 800×g for 5 minutes, counted and re-seeded at a density of 1×10^6 cells per ml. Each flask contains 50–100 ml of fresh medium. The shake flasks were placed on a rotator, incubated at 37° C., and maintained as suspension culture by swirling gently between 90–110 r.p.m. The effect of a polyol such as Pluronic F-68 (0.1%), shown as F-68 below, and copper sulfate (50 nM) on Factor VIII production was examined in shake flasks. Factor VIII was quantitated by a chromogenic assay. The assay is sold commercially as a test kit known as Coatest VIII:C/4 and is available from Baxter HealthCare Products. The cells were maintained by this procedure for 24 days. The Factor VIII activity in each medium, as determined with the Coatest VIII:C/4 kit, is shown in Table 1.

TABLE 1

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal
Basal Medium	0.15 ± 0.07*	0.026 ± 0.013	0.0000
Basal + F-68 (0.1%)**	0.24 ± 0.04	0.052 ± 0.013	200
Basal + F-68 (0.1%) + Cu (50 nM**)	0.42 ± 0.09	0.091 ± 0.013	350

*Mean of 36 samples ± standard deviations. The cells were monitored for Factor VIII production over a period of 24 days as described above.
**Titration experiments showed that 0.1% is the optimal dose for Pluronic F-68. Increasing the concentration to 0.3% had no significant impact on Factor VIII production. Dose-response experiments revealed that 50–800 nM copper sulfate is optimal for Factor VIII production.

As shown in Table 1, the addition of Pluronic F-68 alone or, preferably, in combination with copper sulfate significantly enhanced the titer and specific productivity of BHK cells containing the gene encoding Factor VIII under protein-free conditions.

EXAMPLE 2

To further optimize the production of Factor VIII under protein-free conditions, trace metals were added to the protein-free production medium. Factor VIII production was then assessed by the continuous shake flask culture system as described in example 1 for 16 days. The data is shown in Table 2. In the absence of copper sulfate, the trace metals had no effect on Factor VIII productivity. See Table 2.

TABLE 2

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal + F-68
Basal + F-68	0.46 ± 0.11	0.065 ± 0.013	0.0000
Basal + F-68 + Cu	0.53 ± 0.15	0.078 ± 0.026	120
Basal + F-68 + Cu + metals*	0.73 ± 0.16	0.104 ± 0.026	160

*Metals include $CuSO_4.5H_2O$ (50 nM), $MnSO_4$ (3 nM), $Na_2SiO_3.9H2O$ (1.5 μM), $[NH_4]_6Mo_7O_{24}.4H2O$ (3 nM), $CrK(SO_4)_2.4H_2O$ (1.5 nM), and LiCl (236 nM).

EXAMPLE 3

The effect of trace metals and copper on factor VIII production was further evaluated in a perfusion fermenter. Two 1.5-liter fermenters were seeded with the BHK clonal variant at a density of 2×10^6 cells/ml using the basal medium described in Table 1. The fermenter was perfused at a rate of 0.5 liter/day. One fermenter was kept as a control and the other fermenter was supplemented with copper and trace metals as described in Table 2. The fermenters were maintained for 15 days with an average cell density of ~$2–3\times10^6$ cells/ml. As shown in Table 3, the addition of Pluronic F-68, copper, and trace metals significantly enhanced the specific productivity of BHK cells harboring the gene encoding factor VIII under protein-free conditions under continuous perfusion conditions. This production method can be easily adapted to larger fermenters (200 to 500 liter) equipped with cell retention devices such as settlers.

TABLE 3

Days	Specific Productivity (μU/cell/day) Basal Medium	Cu + metals
1	0.02	0.04
2	0.02	0.05
3	0.02	0.045
4	0.018	0.05
5	0.02	0.05
6	0.035	0.060
7	0.025	0.055
8	0.02	0.04
9	0.025	0.06
10	0.02	0.065
11	0.025	0.070
12	0.025	0.065
13	0.02	0.060
14	0.03	0.06
15	0.02	0.05

Additional Studies

The demonstrated that the expression of Factor VIII in recombinant BHK cells under protein-free conditions is significantly enhanced when cells were cultivated in the presence of polyol and copper sulfate. Factor VIII has been shown to contain a single copper atom, but the role of this metal in the structure and function of Factor VIII remains unclear. Recent reports show that Factor VIII subunits bind copper ions and suggest a functional role for copper in the assembly of the heavy and light chains of Factor VIII (Tagliavacca et al. 1, 1997; Sudhakar et al, 1998). We now further demonstrate that controlled amounts of copper ions alone (without polyols) are able to enhance the productivity of Factor VIII in recombinant BHK cells. Preferably, the copper is provided in the form of copper sulfate at a concentration ranging from about 50 to 800 nM in the culture medium and, very preferably includes the trace ions included in the above examples where polyols were used.

TABLE 4

Effect of Cu^{+2} on the production of FVIII in BHK cells under protein-free conditions.

Conditions	Titer (U/ml)	Specific Productivity (μU/cell/day)	% Increase over basal
Basal Medium	0.14	0.035	0.0000
Basal + Cu^{+2} (50 nM)	0.33	0.082	234
Basal + F-68	0.30	0.078	220
Basal + F-68 + Cu^{+2} (50 nM)	0.54	0.128	365

TABLE 5

Effect of various concentrations of Cu on the production of rFVIII in BHK cells under protein-free conditions.

Concentrations of Cu (nM)	Specific Productivity (μU/cell/day)
Basal Medium	0.040
20	0.062
50	0.078
100	0.080
200	0.082
400	0.080
800	0.081

The above examples are provided as a means of illustrating the present invention and are not to be construed as limiting the invention, which is solely defined by the claims.

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We claim:

1. A method for production of recombinant Factor VIII from mammalian host cells carrying the cDNA coding for Factor VIII, comprising

(a) culturing said mammalian host cells in a medium free of plasma-derived protein and polyol supplements, said medium being supplemented with copper ions; and

(b) culturing for a time and under conditions effective to produce the factor VIII.

2. The method of claim 1 wherein the medium includes copper sulfate in an amount ranging from about 50 to about 800 nM.

3. The method of claim 2 wherein manganese ions are present in an amount ranging from about 1.5 to about 4.5 nM.

4. The method of claim 2 wherein ions containing molybdenum are present in an amount ranging from about 1.5 to about 4.5 nM.

5. The method of claim 2 wherein ions containing silicon are present in an amount ranging from 75 to about 300 nM.

6. The method of claim 2 wherein chromium ions are present in an amount ranging from about 1.0 to about 4.0 nM.

7. The method of claim 2 wherein lithium ions are present in an amount ranging from about 120 to about 480 nM.

8. The method of claim 1 wherein said mammalian host cell is selected from the group consisting of baby hamster kidney cells, human embryonic kidney cells, and Chinese hamster ovary cells.

9. A cell culture medium for the production of recombinant Factor VIII comprising a basal medium free of plasma-derived protein and including copper ions and insulin and which does not include polyols.

10. The medium of claim 9 wherein the copper ions are present in an amount ranging from about 50 to about 800 nM.

11. The medium of claim 10 including at least one trace metal selected from the group consisting of manganese, molybdenum, silicon, chromium and lithium.

* * * * *

EXHIBIT G

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Published
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(54) Title: COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER

(57) Abstract

The present invention relates to novel composition comprising coagulation factor VIII and a non-ionic surfactant such as block copolymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80 as stabilizer. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars or sugar alcohols. The invention also relates to the use of a non-ionic surfactant as stabilizer for a composition comprising coagulation factor VIII.

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COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER.

The present invention relates to a novel formulation comprising coagulation factor VIII and a non-ionic surfactant such as block co-polymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars and/or sugar alcohols.

Haemophilia is an inherited disease which has been known for centuries but it is only within the last three decades that it has been possible to differentiate between the various forms; haemophilia A, haemophilia B and haemophilia C. Haemophilia A is the most frequent form. It affects only males with an incidence of one or two individuals per 10 000 live-born males. The disease is caused by strongly decreased level or absence of biologically active coagulation factor VIII (antihaemophilic factor) which is a protein normally present in plasma. The clinical manifestation of haemophilia A is a strong bleeding tendency and before treatment with factor VIII concentrates was introduced, the mean age of those patients was less than 20 years. Concentrates of factor VIII obtained from plasma have been available for about three decades. This has improved the situation for treatment of haemophilia patients considerably and given them possibility to live a normal life.

Therapeutic factor VIII concentrates have until now been prepared by fractionation of plasma. However, there are now methods available for production of factor VIII in cell culture using recombinant DNA techniques as reported in e.g. J Gitschier et al. Nature 312, 330-37 984 and EP 160 457.

Factor VIII concentrates derived from human plasma contain several fragmented fully active factor VIII forms (Andersson et al, Proc. Natl. Acad. Sci. USA, Vol 83,2979-83, May 1986). The smallest active form has a

molecular mass of 170 kDa and consists of two chains of 90 kDa and 80 kDa held together by a metal ion bridge. Reference is here made to EP 197 901. Kabi Pharmacia has developed a recombinant factor VIII product which corresponds to the 170 kDa plasma factor VIII form in therapeutic factor VIII concentrates. The truncated recombinant factor VIII molecule is termed r-VIII SQ and is produced by Chinese Hamster Ovary (CHO) cells in a cell culture process in serum free medium at finite passage.

The specific activity of r-VIII SQ could be more than 12 000 IU/mg protein and preferably more than 14 000 IU/ mg. Activity of about 15 000 IU/mg has been measured. About 10 000 IU VIII:C per mg protein has earlier been known for our r-VIII SQ.

Recombinant factor VIII SQ is indicated for treatment of classical haemophilia. The dosage is similar to the dosage of the plasma factor VIII concentrates. Due to the high concentration now obtainable only small volumes are needed for injection.

The structure and biochemistry of recombinant factor VIII-products in general have been described by Kaufman Tibtech, Vol 9,1991 and Hematology, 63, 155-65, 1991. The structure and biochemistry of r-VIII SQ have been described in WO 91/09122.

The stability of proteins is generally a problem in pharmaceutical industry. It has often been solved by drying of the protein in different drying processes, such as freeze drying. The protein has thereafter been distributed and stored in dried form.
The solution before drying or freeze-drying, the dried material and the reconstituted product should all be stable, so that not too much activity is lost during the drying process, the storage or during handling.

Factor VIII which has been fractionated from plasma is normally sold as lyophilized powder which should be reconstituted with water.

A formulation with a low amount of protein will generally loose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human

albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang et al., J. of Parenteral Sci. and Tech. Vol 42, Number 2S, supplement. 1988). Human albumin is also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of factor VIII is known and is currently used in all highly purified factor VIII products on the market.
However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.

There is a need for albumin free formulations containing factor VIII and especially recombinant factor VIII which are stable during drying or freeze-drying, in solution and as a solution after reconstitution.

Several solutions have been proposed for the stabilization of different proteins:

EP 35 204 (Cutter) discloses a method for imparting thermal stability to a protein composition in the presence of a polyol.

EP 381 345 (Corint) discloses an aqeous liquid of a peptide, desmopressin, in the presence of carboxymethylcellulose.

In WO 89/09614 (Genentech), a stabilized formulation of human growth hormone comprising glycine, mannitol and a buffer is disclosed and in a preferred embodiment a non-ionic surfactant such as polysorbate 80 is added. The non-ionic surfactant is added for reduced aggregation and denaturation. The formulation has an increased stability in a lyophilized formulation and upon reconstitution.

EP 268 110 (Cetus) discloses a solution comprising a particular protein, interleukin-2, which is dissolved in an inert carrier medium comprising a non-ionic polymeric detergent as a solubilizer/stabilizer. The preferred

detergents are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds and polyethylene sorbitan fatty acid esters.

US 4 783 441 (Hoechst)discloses an aqueous solution comprising a protein, such as insulin and a surface active substance.

US 4 165 370 (Coval) discloses a gamma globulin solution and a process for the preparation thereof. The solutions contains polyethylene glycol (PEG). A non-ionic surfactant can be added to the solution.

In EP 77 870 (Green Cross) the addition of amino acids, monosaccarides, oligosaccarides or sugar alcohols or hydrocarbon carboxylic acid to improve stability of a solution containing factor VIII is disclosed and the addition of sugar alcohol or disaccarides to an aqueous solution of factor VIII for increasing stability during heat treatment has been disclosed in EP 117 064 (Green Cross).

WO 91/10439 (Octopharma) claims stable injectable solution of factor VIII or factor IX which comprises a disaccaride, preferably saccarose and one or more amino acids.

EP 315 968 and EP 314 095 (Rorer) claim stable formulations of factor VIII with different ionic strength.

Proteins are different with regard to physico-chemical properties. When preparing a pharmaceutical preparation which should be physico-chemical acceptable, and stable for a long time, consideration can not only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient and safety for the patient. The results of these aspects are not predictable when testing different formulations and there often is a unique solution for each protein.

In plasma circulating factor VIII is stabilized by association with its carrier protein, the von Willebrand factor (vWF). In plasma and also in conventional intermediate purity factor VIII concentrates the ratio vWF to factor VIII is at least 50:1 on a weight basis. In very high purity factor VIII

concentrates, with a specific activity of more than 2 000 IU per mg protein, the ratio vWF to factor VIII is about 1:1 (w/w) and essentially all factor VIII is bound to vWF. Despite this stabilization further protection by the addition of albumin is required in order to achieve an acceptable stability during lyophilization and storage.

All super pure preparations on the market are stabilized with albumin (human serum albumin).
There is a now a demand for injectable factor VIII without albumin and containing a minimum of additives.

We have now developed a new formulation which solves the above mentioned problems for factor VIII.

To our great surprise we have found that factor VIII, which is a very sensitive protein, can be stabilized without albumin, when a non-ionic surfactant is added.

Thus the present invention relates to a composition comprising a coagulation factor VIII and a non-ionic surfactant as stabilizer. Our factor VIII is highly purified, i.e. has a specific activity of more than 5000 IU/mg protein, and the composition is stabilized without the addition of albumin.
When factor VIII is recombinant it can be either in its full-length form or as a deletion derivative such as SQ derivative.
The amount of factor VIII is from 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.
The non-ionic surfactant is preferably chosen from block co-polymers such as a poloxamer or polyoxyethylene (20) fatty acid ester, such as polysorbate 20 or polysorbate 80. Tween 80® has been used as polysorbate 80.
The non-ionic surfactant should be present in an amount above the critical micelle concentration (CMC). See Wan and Lee, Journal of Pharm Sci, 63, 136, 1974.
The polyoxyethylene (20) fatty acid ester is thus preferably in an amount of at least 0.01 mg/ml. The amount could e.g. be between 0.02 and 1 mg/ml.
The composition can also comprise sodium or potassium chloride, preferably in an amount of more than 0.1 M.

The composition comprises preferably a calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM and an amino acid such as L-histidine in an amount of more than 1 mM. The amount could e.g. be chosen between 0.05 and 500 mM. Mono-or disaccarides such as sucrose or sugar alcohols could be added e.g. in an amount of 1 to 300 mg/ml.

The composition comprises preferably L-histidine and sucrose. The ratio sodium chloride to L-histidine in the composition is preferably more than 1:1.

The composition could comprise
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

To this composition could mono-or disaccarides or sugar alcohols, preferably sucrose be added.
The composition could be in a dried form, preferably lyophilized or in aqeous solution before or after drying. The dried product is reconstituted with sterile water for injection or a buffer solution.

The claimed composition can also be a stable aqeous solution ready for use.

The invention also relates ot compositions in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

The claimed composition can be prepared by mixing factor VIII with a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt or by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

The invention also relates to the use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

An amino acid is used to buffer the system and it protects also the protein in the amorphous phase. A suitable buffer could be L-histidine, lysine and/or arginine. L-Histidine has primarily been chosen because of the good buffer capacity of L-histidine around pH 7.

Sucrose or sugar alcohol can also be added for the protection of the protein.

Calcium (or divalent metal ions), here added as calcium chloride ($CaCl_2$) but other salts such as calcium gluconate, calcium glubionate or calcium gluceptate can also be used, is necessary for the maintenance of the association of factor VIII heavy and light chain.

The data presented in the examples indicate that r-VIII SQ is stable for at least 12 months when stored at 5±3°C.

The following examples illustrate the invention and show stability data for different formulations, all falling under the patent protection, a protection which is not limited to these examples.

The following figures are illustrating the invention:

Figure 1 HPLC gelfiltration, Example 10A, stored 5 months at 25°C.
Figure 2 HPLC gelfiltration, Example 10B, stored 5 months at 30°C.

EXPERIMENTAL
Material and methods

The production of recombinant factor VIII SQ (r-VIII SQ) was essentially performed as described in patent WO 91/09122, example 1-3. A DHFR deficient CHO celline (DG44N.Y.) was electroporated with an expression vector containing the r-VIII SQ gene and an expression vector containing the dihydrofolate-reductase gene. Following selection on selective media surviving colonies were amplified through growth in stepwise increasing amounts of methotrexate. Supernatant from the resulting colonies were individually screened for VIII:C activity. A production clone was chosen and this was subsequently adapted to serum free suspension growth in a defined medium and finally a large scale fermentation process was developed. Supernantant is collected after certain time periods and further purified as described below.

The clarified conditioned medium was pH adjusted and applied to a S-Sepharose FF column. After washing, factor VIII was eluated with a salt buffer containing 5 mM $CaCl_2$.

Immunoadsorption was carried out on an immunoaffinity resin where the ligand was a monoclonal antibody (8A4) directed towards the heavy chain of Factor VIII. Before loading to the column the S-eluate was treated with 0,3 % TNBP and 1 % Octoxynol 9.
The column was equilibrated, washed and factor VIII was eluated with a buffer containing 0,05 M $CaCl_2$ and 50 % ethylene glycol.

The mAb-eluate was loaded on a Q-Sepharose FF column, equilibrated with the elution buffer in the immunoaffinity step. After washing, factor VIII was eluated with 0,05 M L-histidine, 4 mM $CaCl_2$, 0,6 M NaCl, pH 6,8.

The Q-eluate was applied to a gel filtration column (Superdex 200 p.g.). Equilibration and elution was carried out with a formulation containing sodium chloride, L-histidine, calcium chloride and polysorbate 80.

The protein peak was collected and the solution was formulated before freeze drying.

The VIII:C activity and the concentration of the inactive components were adjusted by diluting with an appropriate buffer. The solution was then sterile filtered (0,22 µm), dispensed and freeze-dried. Samples from each composition were frozen and stored at - 70 °C. These samples were thawed and used as references during the assay of VIII:C.

The coagulant activity VIII:C was assessed by a chromogenic substrate assay (Coatest Factor VIII, Chromogenix AB, Mölndal, Sweden). Activated factor X (Xa) is generated via the intrinsic pathway where factor VIII:C acts as cofactor. Factor Xa is then determined by the use of a synthetic chromogenic substrate, S-2222 in the presence of a thrombin inhibitor I-2581 to prevent hydrolysis of the substrate by thrombin. The reaction is stopped with acid, and the VIII:C, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 450 nm against a reagent blank. The unit of factor VIII:C is expressed in international units (IU) as defined by the current International Concentrate Standard (IS) established by WHO.

The recovery of VIII:C is calculated as the percentage of VIII:C in the reconstituted solution divided by the VIII:C in the frozen and thawed solution for freeze-drying with appropriate adjustment for dilutions.

Soluble aggregates were determined by gel filtration. A prepacked Superdex 200 HR 10/30 column (Pharmacia) was used with a fluoroscence detector (exitation wavelength 280 nm), emission wavelength 340 nm). The reconstituted preparation were analysed. Evaluation of results from gelfiltration was done by visual examination of the chromatograms, or by integration of the peak areas if aggregates were found.

Recovery over freeze drying is expressed in % yield of frozen reference.

Example 1. Comparison between albumin and non-ionic surfactant.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

The compositions were the following :

	1A	1B	1C	1D
L-Histidine, mM	50	50	50	50
Sodium chloride, M	0,6	0,6	0,6	0,6
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	-	-	0.02	-
PEG 4000, %	0.1	0.1	-	-
Albumin, %	-	1	-	1
VIII:C charged IU/ml	250	250	250	250
Recovery, IU/ml after reconstit.	83	197	232	222

This example shows that there was no difference in the recovery of factor VIII:C when the non ionic surfactant or albumin was used.

Example 2. Comparison between different strengths of non ionic surfactant

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 2 ml of sterile water for injections.

The compositions were the following :

	2 A	2B	2 C
L-Histidine/L-Glutamate equimolar amount, mg/ml	10	10	10
Sodium chloride, %	2	2	2
Calcium chloride, mg/ml	0.1	0.1	0.1
Polysorbate 80, %	-	0,001	0,01
VIII:C charged IU/ml	300	300	300
Recovery, IU/ml after reconstit.			
Initial	69	133	228
3.5 h*	43	140	222
7h*	49	133	204

* stored as reconstituted solution at ambient temperature

It is here clearly shown the surprisingly good stabilizing effect on factor VIII when a non ionic surfactant is used.

Example 3 , Variation of non-ionic surfactant concentration.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	3 A	3B	3 C	3D	3E
L-Histidine, mM	50	50	50	50	50
Sodium chloride, M	0.34	0.34	0.34	0.34	0.34
Calcium chloride, mM	4	4	4	4	4
Polysorbate 80, %	0.01	0.02	0.03	0.04	0.05
Recovery, after reconstit., %	91	90	93	99	100

Results from this example indicate that the recovery of factor VIII (VIII:C) was very high after reconstitution and good for all concentrations of polysorbate 80 used.

Example 4. Variation of sodium chloride concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized, stored at different temperatures for up to 6 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		4 A	4B
L-Histidine.mM		50	50
Sodium chloride, M		0.3	0.6
Calcium chloride, mM		4	4
PEG-4000 %		0.1	0.1
(Polyethylene glycol)			
Polysorbate 80, %		0.025	0.025
Recovery , %, initial		85	86
stored at 8°C			
	3 mon	88	87
	4 mon	87	83
	6 mon	87	83
stored at 25°C,	1 mon	92	93
	3 mon	87	79
	4 mon	84	81
	6 mon	85	85
stored at 37°C	1 mon	88	90
	3 mon	80	80
	4 mon	80	77
	6 mon	81	80
stored at 50°C	1 mon	84	89
	3 mon	77	77
	4 mon	73	70

0,3 or 0,6 M sodium chloride showed very good stability. Both formulations were stable for 6 months at 37°C.

Example 5. Variation of L-Histidine concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		5 A	5B
L-Histidine, mM		46	59
Sodium chloride, M		0.31	0.31
Calcium chloride, mM		3,7	3,7
PEG-4000 %		0.091	0.091
(Polyethylene glycol)			
Polysorbate 80, %		0.364	0.364
Recovery , %			
stored at 8°C,	Initial	78	84
	3 mon	70	76
stored at 25°C,	1 mon		
	3 mon	69	74
stored at. 37°C	1 mon	76	85
	3 mon	61	48
stored at 50°C	1 mon	60	73
	3 mon	44	48

This example shows that these different amounts of L-histidine does not effect the stability.

Example 6

Recombinant factor VIII was prepared according to the method described under Experimental.

	6A	6B
L-Histidine, mM	65	65
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	4	4
PEG-4000 %	0	0.1
Tween 80, %	0.025	0.025

These solutions were freezed/thawed 1, 5 and 10 times and the recovery was the following:

	IU/ml	IU/ml
cold	298	291
1 freezing	293	293
5	295	287
10	290	288

These studies showed that VIII:C was stable after repeated freeze-thawing and that PEG-4000, which is thought to act as cryoprotectant, is not necessary in this formulation.

Example 7. Variation of pH

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	7 A	7B	7 C	7D
L-Histidine, mM	65	65	65	65
Sodium chloride, M	0,3	0,3	0,3	0,3
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	0.025	0.025	0.025	0.025
pH	6.0	6.5	7.0	7.5
Recovery, %, Initial	74	70	78	79
3 hours*	73	80	78	77

*stored as reconstituted solution at ambient temperature

This example shows that a pH is of no significant importance between 6.0 and 7.5 approx.

Example 8 Addition of sucrose

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	8A	8B
L-Histidine, mM	58	20.5
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	3,7	3,7
Sucrose, mM	0	13.3
Polysorbate 80, %	0.025	0.025

Sucrose was added to the solution B after the final purification step before lyophilization.

The recovery after freeze-drying was 76 % for A and 87 % for B. The same activity was found 4 hours after reconstitution stored at room temperature.

This study indicated that the addition of sucrose is favourable for the recovery of VIII:C over freeze-drying.

Example 9 . Variation of calcium salt

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	9 A	9B	9C	9D
L-Histidine, mM	23	23	23	23
Sodium chloride, M	0,34	0,34	0.34	0,34
Calcium chloride, mM	4	4	0.15	0.15
Polysorbate, %	0.025	0.025	0.025	0.025
Sucrose, mM	-	10	-	10
Calciumgluconate, mM	0	0	6	6
Recovery,%, Initial	63	74	74	78
4 hours*	60	73	73	77

*stored as reconstituted solution at ambient temperature

This example shows that $CaCl_2$ can be substituted by Calcium gluconate.

Example 10

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 1000 IU.

	10A	10B
L-Histidine, mM	14.7	58
Sodium chloride. M	0.31	0.31
Calcium chloride, mM	3.7	3.7
Sucrose, mM	19.9	-
Polysorbate 80, %	0.025	0.025
Recovery, IU/ml after reconstitution		
Initial	213	198
4 h, 25 °C	213	198
24, 25 °C	201	182
Recovery , %		
Initial	92	91
5 months, 25°C	88	-
5 months, 30°C	76	85
12 months, 7°C	89	97

The recovery was good when part of the L-histidine was substituted by sucrose.

These formulations were studied by gelfiltration after 5 months´storage at 25°C and 30°C, respectively and the results are shown in figures 1 and 2. The only peaks to be seen is the peak at 42, indicating factor VIII:C and the peak at 70 which is histidine. Aggregates is to be found earlier than 40. From figure 1 it can be seen that no detectable amount of aggregates was found after 5 months at 25°C for 10A. Figure 2 shows a small amount of aggregates which is less than 2 % after 5 months at 30°C for 10B .

Example 11

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

	11A	11B
L-Histidine, mM	14.7	58
Sodium chloride, M	0.31	0.31
Calcium chloride, mM	3.7	3.7
Sucrose, mM	19.9	-
Polysorbate 80, %	0.025	0.025
Recovery, IU/ml after reconstitution		
Initial	98	105
4 h, 25 °C	96	103
24, 25°C	93	101
Recovery , %		
Initial	91	93
stored at 25°C, 5 mon	89	87
stored at 30°C, 5 mon	76	79
stored at 7°C 12 mon	88	89

Both formulations showed good stability.

These formulations were studied by gelfiltration and the results were similar as shown in Figures 1 and 2.

No aggregation was formed when the formulations had been stored for 5 months at 25°C and 30°C, respectively.

Example 12

Recombinant factor VIII was prepared according to the method described under Experimental.

2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 4 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

	12A	12B
Mannitol, mg/ml	20	20
L-Histidine, mg/ml	2,67	2.67
Sodium chloride, mg/ml	18	18
Calcium chloride, mM	3,7	3,7
Polysorbate 80, mg/ml	0.23	0.23
Recovery , %		
initial	91	93
stored at. 7°C 5 mon	90	85

An acceptable stability was achieved after five months at 7°C.

CLAIMS

1. A composition comprising coagulation factor VIII and a non-ionic surfactant as stabilizer.

2. A composition according to claim 1 in which factor VIII is highly purified and stable without the addition of albumin.

3. A composition according to claim 1 or 2 in which factor VIII is full-length or a deletion derivative of recombinant factor VIII.

4. Composition according to any of claims 1-3 in which the amount of factor VIII is 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.

5. Composition according to any of claims 1-4 in which the non-ionic surfactant is present in an amount above the critical micelle concentration.

6. Composition according to any of claims 1-5 in which the non-ionic surfactant is chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80.

7. Composition according to claim 6 in which the polyoxyethylene (20) fatty acid ester is in an amount of at least 0.01 mg/ml.

8. Composition according to any of claims 1-7 which comprises sodium or potassium chloride, preferably in an amount of more than 0.1 M.

9. Composition according to any of claims 1-8 which comprises calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.

10. Composition according to any of claims 1-9 which comprises an amino acid such as L-histidine in an amount of more than 1 mM.

11. Composition according to any of claims 1-10 which comprises mono-or disaccarides, prferably sucrose or sugar alcohols.

12. Composition according to any of claims 10-11 which comprises L-histidine and sucrose.

13. Composition according to claim 8 and 10 in which the ratio sodium chloride to L-histidine is more than 1:1.

14. Composition according to any of claims 1-13, comprising
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

15. Composition according to any of claims 1-14 which is dried.

16. Composition according to claim 15 which is lyophilized.

17. Composition according to any of claims 1-14 which is in a stable aqeous solution ready for use.

18. Composition according to any of claims 3-17 in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

19. Process for the preparation of the composition according to claim 1 characterized by mixing factor VIII with a non ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

20. Process for the preparation of the composition according to claim 1 characterized by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably

together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

21. Use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

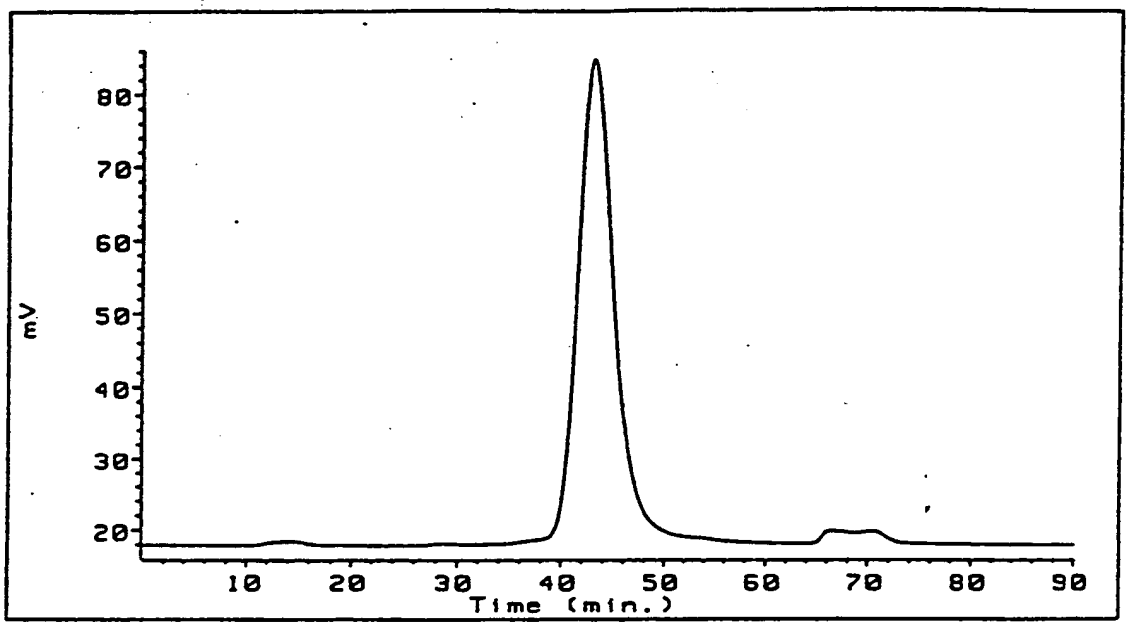

Figure 1

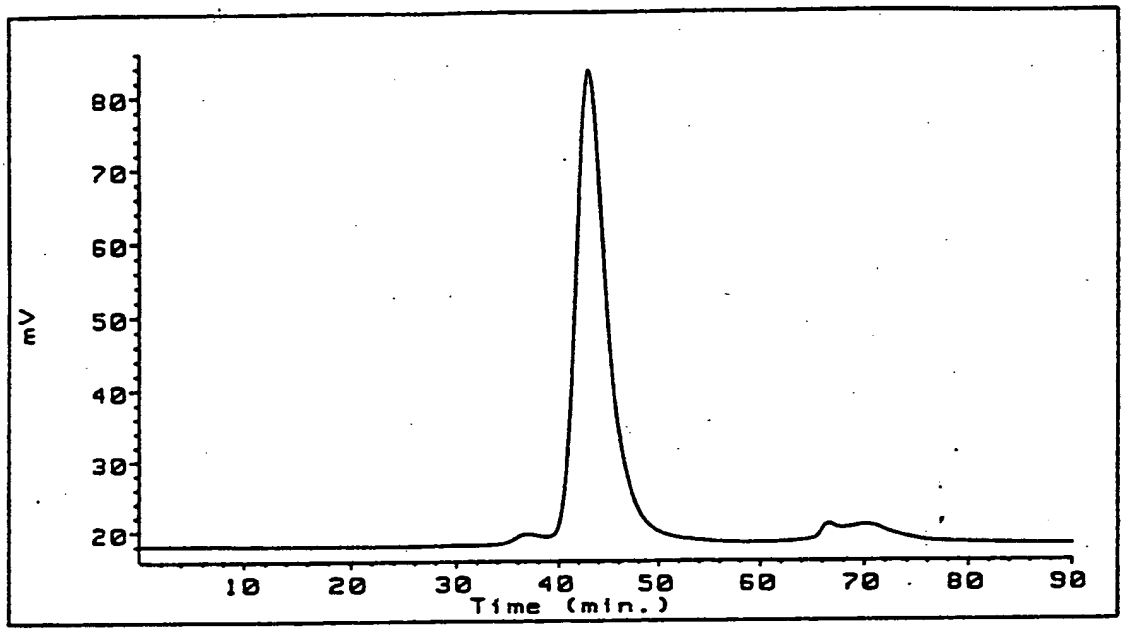

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PC.. SE 93/00793

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 35/16, A61K 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP, A1, 0508194 (BEHRINGWERKE AG), 14 October 1992 (14.10.92), see claim 6, examples 1-2	1-21
X	EP, A3, 0099445 (NEW YORK BLOOD CENTER, INC.), 1 February 1984 (01.02.84), see page 8, line 7 - line 14; page 19, line 24 - page 20, line 27	1-21
A	WO, A1, 9110439 (OCTA PHARMA AG), 25 July 1991 (25.07.91)	1-21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
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Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1994	12 -01- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

Information atent family members

27/11/93

International application No.
/SE 93/00793

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